

Affinity Labeling of a Tyrosine Residue in the ATP Binding Site of the *recA* Protein from *Escherichia coli* with 5'-*p*-Fluorosulfonylbenzoyladenosine*

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We have covalently modified the *recA* protein from *Escherichia coli* with the adenine nucleotide analog 5'-*p*-fluorosulfonylbenzoyladenosine (5'-FSBA). The rate at which the protein is modified shows a sigmoidal dependence on the concentration of 5'-FSBA suggesting that binding of the analog is characterized by positive cooperativity. Covalent modification of the protein results in irreversible inactivation of its single-stranded DNA-dependent ATPase activity such that 100% inactivation is achieved when 25% of the enzyme monomers have been modified. Attachment of 5'-FSBA is specific for the ATP-binding site of *recA* protein as judged by the following criteria: (i) attachment of the affinity label to the protein appears to saturate at 1 mol of 5'-FSBA/mol of protein; (ii) binding of 5'-FSBA to *recA* protein is inhibited by ATP and competitive inhibitors of its ATP hydrolytic activity, *e.g.* adenosine-5'-*O*-(thiotriphosphate), ADP, UTP, and GTP, but not by adenosine; (iii) attachment of 5'-FSBA to the protein occurs at a single site as determined by high pressure liquid chromatography peptide separation. Following trypsin digestion of *recA* protein that had been covalently modified with [³H]5'-FSBA we isolated a single labeled peptide (T31) containing the exclusive site of 5'-FSBA attachment. A secondary proteolytic digestion was performed on both 5'-FSBA modified T31 and unmodified T31 using *Staphylococcus aureus* V8 protease, and by comparison of the amino acid compositions of the resulting peptides we identified Tyr-264 as the exclusive site of 5'-FSBA attachment in *recA* protein.

The *recA* protein of *Escherichia coli*, a polypeptide with a molecular weight of approximately 38,000, possesses two remarkably dissimilar types of activities. First, the enzyme promotes hybridization between complementary single-stranded DNA molecules (1) or between single strands and homologous duplex partners (2, 3). ATP is required as a cofactor in these reactions and is hydrolyzed by *recA* protein to ADP and P_i. Second, *recA* protein, together with polynucleotide and ATP, promotes the proteolytic cleavage of phage λ repressor (4), and the cellular *lexA* repressor (5) which controls expression of the SOS functions, including enhanced DNA repair, mutagenesis and regulation of cell division. In these latter reactions, unlike the hybridization activities, ATP can be replaced with the nonhydrolyzable analog

ATPγS¹ indicating that ATP binding but not its hydrolysis is important for repressor inactivation (6). Binding studies have demonstrated that ATPγS traps *recA* protein in a stable ternary complex with DNA and the analog (7) whereas ATP promotes dissociation of *recA* protein and single-stranded DNA (8). These and other results argue that interaction of ATP with *recA* protein has important consequences for the structure and function of this enzyme.

Recently, we have used the photoaffinity ATP analog 8-azidoadenosine 5'-triphosphate (N₃-ATP) to covalently modify *recA* protein (9). The observations that this analog was efficiently hydrolyzed by *recA* protein in a DNA-dependent reaction and that photolabeling was blocked by substrates and competitive inhibitors of ATP hydrolysis indicated that this analog recognized and covalently modified the ATP binding domain of *recA* protein. A single tryptic fragment spanning residues 257-280 in the protein sequence that contained the site of covalent attachment of N₃-ATP was isolated from photolabeled protein. In the accompanying paper (23) we demonstrate that Tyr-264 is the unique site of N₃-ATP attachment in this peptide.

It seemed reasonable to us that the utility of N₃-ATP may be restricted to modifying and identifying amino acid residues that are in close proximity to the adenine ring of ATP rather than those residues that participate in phosphodiester bond hydrolysis *per se*. In order to identify the region of *recA* protein that is likely to contain residues involved in catalysis we have used another ATP analog, 5'-FSBA, as a probe for the hydrolytic site on the protein. The structure of 5'-FSBA is such that its reactive sulfonyl fluoride moiety may be positioned similarly to the γ-phosphate of ATP. The electrophilic sulfonyl fluoride group can react with several types of amino acid residues within enzyme binding sites to form a stable covalent adduct resulting in the irreversible inactivation of enzyme activity. Examples of this include the reaction of 5'-FSBA with both lysyl and tyrosyl residues within two sites on yeast pyruvate kinase (10), a specific tyrosine residue within the nucleotide binding site of the β-subunit of beef heart mitochondrial ATPase (11), a lysyl residue within the coenzyme binding site of pig heart mitochondrial malate dehydrogenase (12), specific lysyl and tyrosyl residues within the NADH inhibitory site of bovine liver glutamate dehydrogenase (13), and a specific lysine residue within the ATP-binding site of *E. coli* glutamine synthetase (14). By way of contrast, a novel form of reversible inactivation of enzyme activity has been demonstrated following reaction of 5'-FSBA with cysteine residues within the active site of rabbit muscle

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¹ The abbreviations used are: ATPγS, adenosine 5'-*O*-(thiotriphosphate); N₃-ATP, 8-azidoadenosine 5'-triphosphate; 5'-FSBA, 5'-*p*-fluorosulfonylbenzoyladenosine; HPLC, high-pressure liquid chromatography.

myosin subfragment I (15) and rat liver *S*-adenosylhomocysteinase (16). The properties of 5'-FSBA and its use as a purine nucleotide-binding site probe have recently been reviewed by Colman (17).

In this report we demonstrate that 5'-FSBA forms a stable covalent modification of *recA* protein resulting in irreversible inactivation of the enzyme's ATPase activity. The incorporation of 5'-FSBA into *recA* protein approaches 1 mol of 5'-FSBA/mol of protein and is severely reduced when reactions are carried out in the presence of substrates or competitive inhibitors of the *recA* protein nucleoside triphosphatase activity. These results demonstrate that attachment of 5'-FSBA is specific for the ATP hydrolytic site of *recA* protein. Using HPLC we have purified a tryptic peptide containing the unique site of 5'-FSBA attachment. Further digestion of this peptide with *Staphylococcus* V8 protease and amino acid analysis of the resulting peptides demonstrate that Tyr-264 is the exclusive site of covalent attachment of 5'-FSBA to *recA* protein. These results together with those of the accompanying paper (23) suggest a model for binding of ATP to the active site of *recA* protein. Moreover, the covalent modification of Tyr-264 by both N_3 -ATP and 5'-FSBA indicates that this is a particularly reactive residue that may be critical for the mechanism of ATP hydrolysis by *recA* protein.

EXPERIMENTAL PROCEDURES

Materials—*recA* protein was purified to homogeneity from the *E. coli* K12 strain KM1842 using ATP elution from single-stranded DNA-cellulose as previously described (18). Protein concentrations were determined using a value of $\epsilon_{280}^{1\%} = 5.16$ (19). The numbering of amino acid residues is that of Sancar *et al.* (20). [3H]5'-FSBA was prepared as described (21) using [2,8- 3H]adenosine (ICN) as a precursor. Hexamethylphosphoric triamide and *p*-fluorosulfonylbenzoylchloride were purchased from Aldrich. The specific activity of the resulting product was 1.8 Ci/mol. The purity of the [3H]5'-FSBA was determined by thin-layer chromatography using cellulose plastic-backed sheets (Eastman 13255) and a solvent containing 2-butanol:acetic acid:water (200:30:75). The standards used were 5'-FSBA obtained from Sigma and [3H]5'-FSBA, a generous gift from Dr. Emil Reisler (Molecular Biology Institute, UCLA). The concentration of 5'-FSBA solutions in 100% ethanol was determined using values of $\epsilon_{232} = 1.88 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{259} = 1.35 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (21). Stock solutions of 5'-FSBA were prepared in dimethylformamide immediately before use. ATP, ADP, and adenosine were purchased from Sigma. Trypsin was purchased from Worthington and *S. aureus* V8 protease was purchased from Miles.

Modification of *recA* Protein with 5'-FSBA—The reaction of *recA* protein with 5'-FSBA was performed at 30 °C in a buffer containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 20 mM KCl. The concentrations of *recA* protein and 5'-FSBA are indicated in the figure and tables and varied from 20 to 50 μM and 100 μM to 2.5 mM, respectively. Measurements of covalent attachment of [3H]5'-FSBA to *recA* protein were performed using the chromatographic assay of Penefsky (22). Aliquots were removed from reaction mixtures at indicated times and loaded onto 1.0-ml Sephadex G-50 columns prepared in disposable plastic syringes. Following brief centrifugation (4 min at 1600 $\times g$) the entire volume of effluent containing the labeled protein was used for scintillation counting.

For preparative labeling experiments *recA* protein was modified with 5'-FSBA, dialyzed exhaustively against 0.1 M NH_4HCO_3 (pH 7.2), and reduced and carboxymethylated as previously described (9) with the following alteration. Upon completion of carboxymethylation the protein was separated from small molecules by gel filtration on Sephadex G-25 equilibrated in 0.1 M NH_4HCO_3 (pH 7.2), rather than by prolonged dialysis. Following preparative labeling of *recA* protein with 5'-FSBA all subsequent steps (dialysis, proteolysis, and HPLC purification of peptides) were performed in solutions buffered to pH 7.0 or as close to it as possible. Because slightly alkaline conditions were required for carboxymethylation, reactions were carried out in as short a time as possible, typically 40 min. These modifications were designed to minimize losses of label due to spontaneous hydrolysis of the ester linkage between the adenosine and *p*-fluorosulfonylbenzoyl moieties in 5'-FSBA. Esch and Allison (11)

have shown that the half-life of this linkage is 3.6 days at pH 8.0 and 23 °C.

ATPase Assay—The single-stranded DNA-dependent ATP hydrolytic activity of *recA* protein was assayed as previously described (19).

Proteolytic Digestions/HPLC Purification of Peptides—Digestions with trypsin and *S. aureus* V8 protease, and HPLC purification of peptides were performed as described in the accompanying paper (23). Amino acid analysis was performed as previously described (9).

RESULTS

Covalent Attachment of 5'-FSBA to *recA* Protein—Incubation of *recA* protein (25 μM) and 5'-FSBA (100 μM) resulted in a relatively slow rate of modification that was linear for more than 60 min (Fig. 1A). We measured the rate of covalent attachment at several higher 5'-FSBA concentrations and observed that the rate of labeling was strongly concentration-dependent. As shown in Fig. 1A less than 30% of the protein was modified after 60 min in the presence of 700 μM 5'-FSBA. However, by increasing the concentration to 1.5 mM we could modify more than 75% of the *recA* protein after 60 min. A

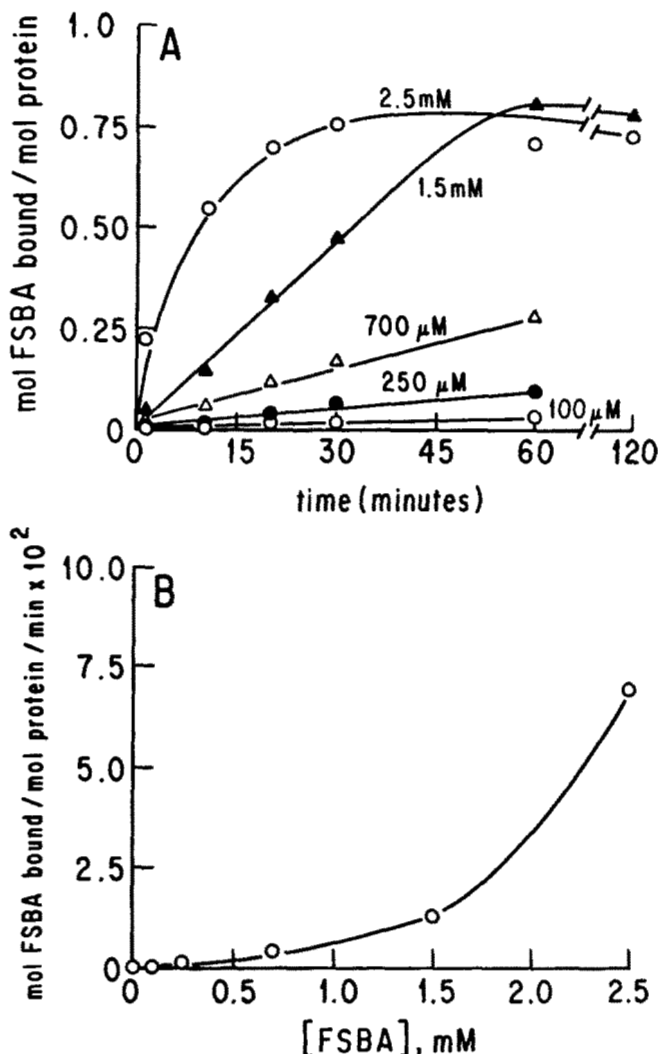


FIG. 1. Cross-linking of 5'-FSBA to *recA* protein. A, reactions (380 μl) contained 25 μM *recA* protein and the indicated concentrations of 3H -labeled 5'-FSBA. Other reaction components and the assay for covalent attachment of 5'-FSBA to *recA* protein are described under "Experimental Procedures." Aliquots (60 μl) were withdrawn for assay at each time point. B, the initial rate of 5'-FSBA cross-linking to *recA* protein was calculated for each of the time courses in A and plotted as a function of the concentration of 5'-FSBA.

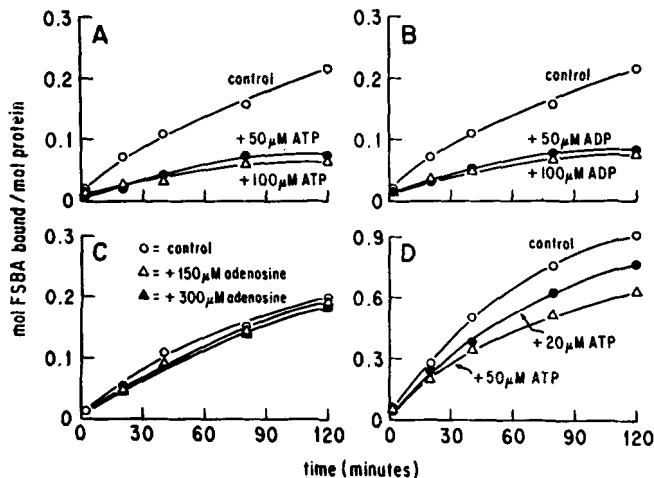


FIG. 2. Inhibition of 5'-FSBA cross-linking to *recA* protein. Reactions (350 μ l) contained 25 μ M *recA* protein, the indicated concentrations of ATP, ADP, or adenosine, and either 200 μ M (A-C) or 1.0 mM (D) 3 H-labeled 5'-FSBA. Aliquots (60 μ l) were withdrawn for assay at each time point. Other reaction components and the assay procedure are described under "Experimental Procedures."

further increase to 2.5 mM 5'-FSBA resulted in a rapid rate of labeling which reached a limit of approximately 0.8 mol of 5'-FSBA bound per mol of enzyme. In other experiments where either the protein or the 5'-FSBA concentrations were varied we observed that the maximum incorporation of 5'-FSBA into *recA* protein approached 1.0 mol of 5'-FSBA bound per mol of protein (data not shown). Incubation of [3 H] 5'-FSBA-labeled *recA* with 5 mM dithiothreitol resulted in no change in the amount of label bound to the protein (data not shown). The rate of 5'-FSBA attachment was determined from the linear portion of the curves shown in Fig. 1A and plotted against the 5'-FSBA concentration (Fig. 1B). We were unable to increase the concentration of 5'-FSBA beyond 2.5 mM due to the appearance of precipitation (see "Discussion"). However, over the concentration range examined the rate of 5'-FSBA labeling displayed an apparent sigmoidal dependence on 5'-FSBA concentration.

Specificity of 5'-FSBA Interaction with *recA* Protein—The specificity of the interaction between 5'-FSBA and *recA* protein was determined by measuring the rate of covalent modification in the presence of ATP, ADP, and adenosine. The *recA* protein ATPase activity is competitively inhibited by ADP (24) but is not inhibited by adenosine.² Fig. 2 shows the results of experiments in which 25 μ M *recA* protein was reacted with 300 μ M 5'-FSBA in the presence of either ATP (panel A) or ADP (panel B). Both compounds significantly decrease the rate of 5'-FSBA incorporation into *recA* protein. We estimate from these data that the rate of incorporation of 5'-FSBA is reduced approximately 50% when the concentration of either ATP or ADP is 30 μ M. This value is consistent with previously determined steady-state kinetic values of *recA* protein DNA-dependent ATPase activity: K_m for ATP is 20 μ M and K_i for ADP is approximately 15 μ M (7, 24). Similar results were obtained when reactions were performed in the presence of GTP, a competitive inhibitor of the *recA* protein ATPase activity (data not shown). The concentration of GTP required for 50% reduction in the rate of 5'-FSBA incorporation (80 μ M) is again compatible with its K_i derived from steady-state analysis of *recA* protein ATPase activity (60 μ M) (24). When modification reactions were carried out in the presence of equivalent concentrations of adenosine no de-

crease in the rate of incorporation of 5'-FSBA into protein was observed (Fig. 2, panel C). This result is consistent with the inability of adenosine to inhibit the ATPase activity of *recA* protein. Specific attachment of the photoaffinity label N_3 -ATP to *recA* protein is also efficiently reduced by competitive inhibitors of the *recA* protein ATPase activity, but not by adenosine (9). These results indicate that 5'-FSBA attachment to *recA* protein has characteristics in common with the photolabeling of the ATP active site.

The results presented in Fig. 2, panel D, indicate that in the presence of relatively low concentrations of ATP there is a significant reduction in the rate of 5'-FSBA incorporation into *recA* protein even at a high 5'-FSBA concentration. When *recA* protein is incubated with 1.26 mM 5'-FSBA the rate of incorporation is reduced by 30% in the presence of 20 μ M ATP and by 50% when the ATP concentration is increased to 50 μ M. These results suggest that although 5'-FSBA attachment occurs specifically within the ATP-binding site, the affinity of *recA* protein for this analog is considerably lower than for ATP.

That *recA* protein has a low binding affinity for 5'-FSBA relative to ATP is supported by the data presented in Table I. In these experiments we tested the ability of 5'-FSBA to inhibit the single-stranded DNA-dependent ATPase activity of *recA* protein when present with ATP in the hydrolysis reaction. Very little reduction in the rate of ATP hydrolytic activity was observed in the presence of 5'-FSBA. Even under conditions where 5'-FSBA was present in a 20-fold excess over ATP (ATP = 76 μ M, 5'-FSBA = 1.5 mM) no significant decrease in the rate of ATP hydrolysis was detected. We were prevented from using higher concentrations of 5'-FSBA in these experiments because at 5'-FSBA concentrations greater than 2.0 mM the reaction mixtures showed a variable amount of precipitation. Under the conditions used to measure ATP hydrolysis the extent of covalent modification of *recA* protein by 5'-FSBA is less than 3%.

Inactivation of *recA* Protein ATPase by Preincubation with 5'-FSBA—Following modification of *recA* protein with 5'-FSBA the single-stranded DNA-dependent ATPase activity became partially and irreversibly inactivated. Incubation of modified protein with 5 mM dithiothreitol had no effect on this inactivation (data not shown). We performed experiments in order to correlate the degree of covalent modification of *recA* protein by 5'-FSBA with the degree of inactivation of its ATPase activity. Aliquots were removed from cross-linking reactions at specific times for determination of the degree of covalent protein modification and the rate of ATP hydrolysis.

TABLE I

5'-FSBA as an inhibitor of *recA* protein ATP hydrolytic activity

Reaction conditions and assay procedures are described under "Experimental Procedures." Reactions (60 μ l) contained 2 μ M *recA*, 21 μ M single-stranded M13 DNA, and the indicated concentrations of both [3 H]ATP and 5'-FSBA. Turnover numbers were calculated from initial rate determinations of ATP hydrolysis made during the early portion of the time course; within 3 min for [ATP] = 180 μ M and within 1.5 min for [ATP] = 76 μ M. Covalent modification of *recA* protein by 5'-FSBA under these conditions is less than 3%.

[ATP]	[5'-FSBA]	Turnover No.
μ M	mM	mol ADP formed/mol protein/min
180	0	7.8
180	0.63	7.8
180	1.23	7.5
76	0	8.6
76	0.75	8.6
76	1.50	7.5

² K. Knight, unpublished results.

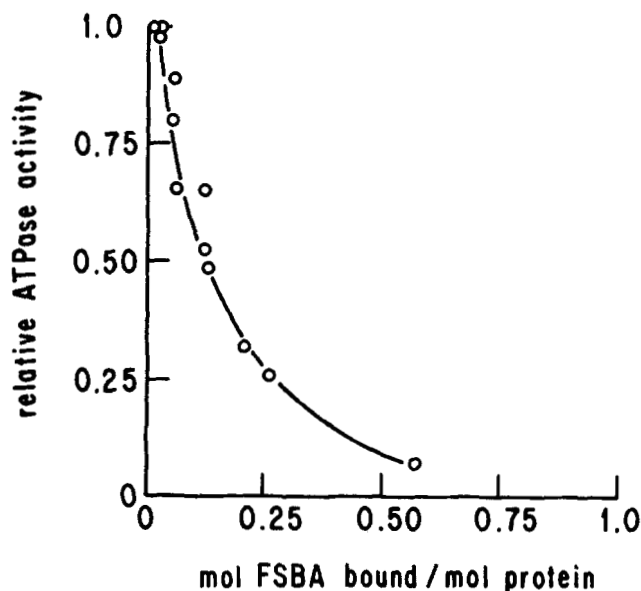


FIG. 3. ATP hydrolytic activity of *recA* protein is inactivated by cross-linking with 5'-FSBA. Cross-linking reactions (350 μ l) contained 20 μ M *recA* protein and varying concentrations of 3 H-labeled 5'-FSBA (200 μ M–1.4 mM). During the course of the reactions aliquots were withdrawn for assay of both covalent attachment of 5'-FSBA to *recA* protein (60 μ l) and the single-stranded DNA-dependent ATP hydrolytic activity of *recA* protein (6 μ l). ATPase reactions (60 μ l) contained 2 μ M *recA* protein, 0.5 mM [3 H]ATP (67 Ci/mmol), and 21 μ M single-stranded M13 DNA. All points represent an initial rate determination for the ATPase activity using *recA* protein that had been modified by 5'-FSBA to different extents. Additional reaction components and assay conditions are described under "Experimental Procedures."

From the results shown in Fig. 3 it is clear that the extent of inactivation of the *recA* protein ATPase activity is greater than the extent of protein modification. Extrapolation of the linear portion of this inactivation curve to a relative activity equal to 0 falls close to 0.25 mol of 5'-FSBA bound per mol of protein. We have observed a qualitatively similar relationship between the degree of photolabeling of *recA* protein with N_3 -ATP and the degree of inactivation of its ATPase activity (9). This nonlinear inactivation curve could result in part from the requirement for a multimeric form of the *recA* protein for ATP hydrolysis (24, 25). The formation of oligomers of *recA* protein containing both modified and unmodified monomers might result in the inactivation of the entire oligomer and produce a larger degree of inactivation of ATPase activity than would be expected for a given amount of protein modification.

HPLC Purification of 5'-FSBA-labeled Tryptic Peptide from *recA* Protein—In order to determine whether 5'-FSBA modification had occurred at more than one site and to identify these regions, we labeled *recA* protein with [3 H]5'-FSBA and subjected it to trypsin digestion. Peptides resulting from tryptic digestion of [3 H]5'-FSBA-labeled *recA* protein were analyzed by HPLC on a C-18 reverse-phase column. The eluent was monitored at 254 nm as well as at 229 nm in order to aid in the detection of the adenine moiety of 5'-FSBA-modified peptides. Aliquots of all fractions were withdrawn for determination of 3 H label by liquid scintillation counting. Greater than 90% of the radioactivity applied to the column was recovered in the profile shown in Fig. 4. Approximately 40% of the 3 H label was contained in fractions eluting at minute 23 and approximately 60% of the label was contained in fractions eluting in minutes 71–73. The radioactive peaks

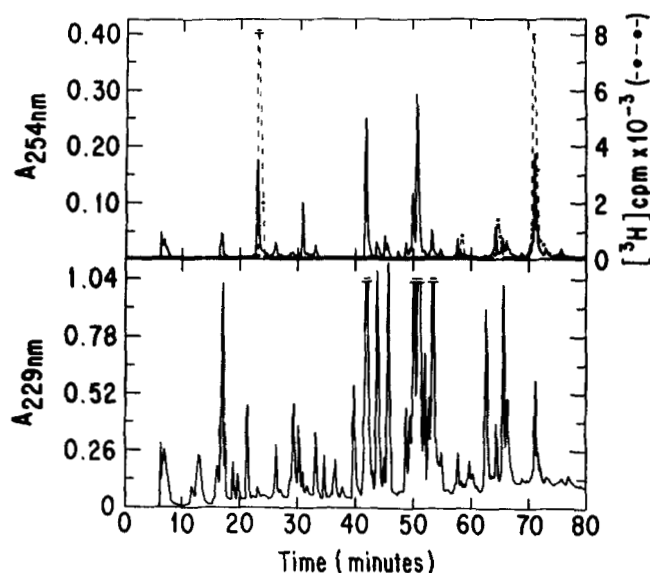


FIG. 4. C-18 HPLC profile of tryptic peptides of *recA* protein following cross-linking with 5'-FSBA. Tryptic peptides from 250 nmol of *recA* protein following cross-linking with 3 H-labeled 5'-FSBA were separated using a Vydac C-18 reverse-phase column eluted at a flow rate of 2.0 ml/min. The profile was developed with a linear gradient of solvent B (70% CH_3CN) into solvent A (20 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.8), 0–91 min (0–65% B). Fractions were collected every 0.5 min and aliquots (40 μ l) were withdrawn for assay of 3 H counts/min. Fractions containing radioactivity near minute 23 and minute 71 were pooled separately and lyophilized.

eluting early and late exhibit a high absorbance at 254 nm indicating the presence of the adenine ring whereas only the late eluting peak had an appreciable absorbance at 229 nm. The radioactive material eluting at minute 23 showed no associated peptide material upon amino acid analysis and was subsequently determined to be free [3 H]adenosine by two criteria: a sample of this material co-migrated with a [3 H]adenosine standard upon TLC analysis using a cellulose thin-layer plate and 2-butanol:acetic acid:water (200:30:75) as solvent. Additionally, a [3 H]adenosine standard eluted at minute 23 when chromatographed on the same C-18 reverse phase column run under gradient conditions identical to those described in Fig. 4. The appearance of free [3 H]adenosine at this step in the purification likely results from spontaneous hydrolysis of the ester bond in 5'-FSBA. (see "Experimental Procedures").

The labeled peptide material eluting in minutes 71–73 was pooled and further purified by HPLC on a C-4 reverse-phase column. As shown in Fig. 5 a single major peak containing approximately 90% of the applied radioactivity eluted in minutes 74–76. Smaller peaks representing minor contaminants eluted in minutes 50–70. As expected, the major peak also had an appreciable absorbance at 254 nm indicative of covalent modification by 5'-FSBA. No other peaks were observed at other positions in the gradient. We determined the amino acid composition of this major peak of peptide material. The results of this analysis (Table II) show a composition essentially identical to tryptic peptide T31 in *recA* protein, spanning residues 257–280. The sequence of this peptide is Gln 257-Ala-Glu-Phe-Gln-Ile-Leu-Tyr-Gly-Glu-Gly-Ile-Asn-Phe-Tyr-Gly-Glu-Leu-Val-Asp-Leu-Gly-Val-Lys-280. The only discrepancy between the composition of the 5'-FSBA-linked peptide and that of peptide T31 is that only 1 mol of Tyr is detected per mol of peptide (Table II). Based on the recoveries of 3 H label and peptide material from this peak we calculated a ratio of approximately 0.9 mol of 5'-FSBA bound

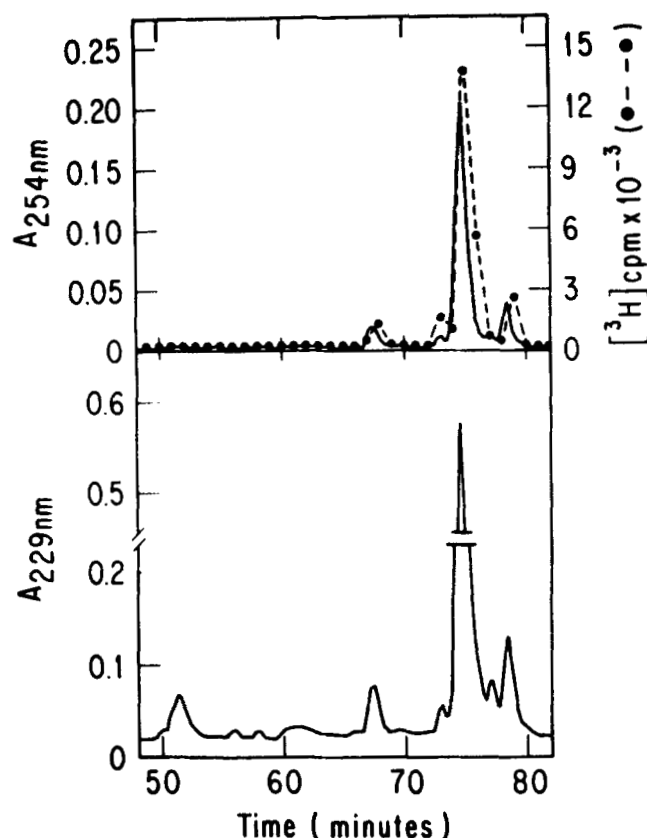


FIG. 5. C-4 HPLC purification of 5'-FSBA-labeled tryptic peptide. Radioactive peptide material purified by C-18 HPLC separation of tryptic peptides (see Fig. 4, minute 71) was further purified using a Vydac C-4 reverse-phase column eluted at a flow rate of 1.0 ml/min. The profile was developed with a linear gradient of solvent B (70% CH₃CN) into solvent A (20 mM NaH₂PO₄/Na₂HPO₄, pH 6.8), 0–10 min (0–25% B), 10–110 min (25–45% B). Fractions were collected every 1.0 min and aliquots (25 μ l) were withdrawn for assay of ³H counts/min. Fractions containing the large peak of radioactivity (74–76) were pooled and lyophilized.

TABLE II

Amino acid composition of the 5'-FSBA-labeled tryptic peptide

The major peak of absorbance and radioactivity in the C-4 HPLC profile presented in Fig. 5 was analyzed for amino acid composition (5'-FSBA/T31). The lane marked T31 is the composition of tryptic peptide T31 (residues 257–280) as determined from the known protein sequence (20). Experimentally determined values are the average of duplicates and have been normalized to those expected from the known amino acid composition of T31.

	5'-FSBA/T31	T31
Aspartic acid	2.0	2
Threonine		
Serine		
Glutamic acid	5.3	5
Proline		
Glycine	4.6	4
Alanine	1.1	1
Valine	2.2	2
Methionine		
Isoleucine	1.8	2
Leucine	2.9	3
Tyrosine	1.2	2
Phenylalanine	2.1	2
Histidine		
Lysine	0.8	1
Arginine		
CM-cysteine		

per mol of peptide. In earlier experiments we had located the position of the unmodified T-31 peptide near minute 63 in the C-18 HPLC profile (9). By comparing this earlier profile with the elution profile shown in Fig. 4, we located the underivatized T31 peptide at minute 66. This material was isolated, further purified by HPLC on a C-4 column, and subjected to amino acid analysis. This analysis gave a composition expected of peptide T31 with the recovery of 2 mol of Tyr/mol of peptide (data not shown). Thus, the poor yield of Tyr recovered from the 5'-FSBA-linked peptide results from modification since in the same gradient we can recover unmodified T31 peptide containing both Tyr residues. A similar result was obtained when we used N₃-ATP to covalently modify *recA* protein (9). These results suggested that either Tyr-264, Tyr-271, or both residues could be the site of covalent attachment of 5'-FSBA.

Identification of the Site of Attachment of 5'-FSBA within T31—Previously, we presented evidence that purified T31 contains a glutamine residue at the NH₂ terminus that readily cyclizes to form a nonreactive pyrrolidone ring thereby blocking direct sequencing of the peptide (9). Therefore, in order to localize the site(s) of 5'-FSBA attachment within the peptide we performed a secondary proteolytic digestion to obtain shorter labeled peptides that would either be amenable to sequence analysis or would yield an unambiguous amino acid composition. We chose *S. aureus* V8 protease, which cleaves at glutamyl peptide bonds, for the secondary proteolytic digestion of 5'-FSBA-labeled T31 because cleavage at Glu-266 would separate Tyr-264 and Tyr-271 into two 7-residue peptides. The sequences of the expected peptides resulting from *S. aureus* V8 protease cleavage of T31 are presented in Fig. 6.

In control experiments we digested samples of underivatized T31 (purified as described above) with *S. aureus* V8 protease as described under "Experimental Procedures" and separated the peptides by HPLC on a C-4 reverse column (Fig. 7). Three clearly resolved peaks (a, b, and c) eluting early in the gradient were isolated and the amino acid composition of each peak was determined (Table III). The composition of peak a (21 min) corresponds to peptide S3, residues 267–273, that of peak b (23 min) corresponds to peptide S4, residues 274–280, and that of peak c (25 min) corresponds to peptide S2, residues 260–266. Peptides S2, S3, and S4 were recovered in approximately equimolar amounts and all amino acid residues were recovered in the expected molar yield. The 3-residue S1 peptide (residues 257–259) was apparently not retained on this column.

Following digestion of 5'-FSBA-modified T31 with *S. aureus* V8 protease the resulting peptides were chromatographed

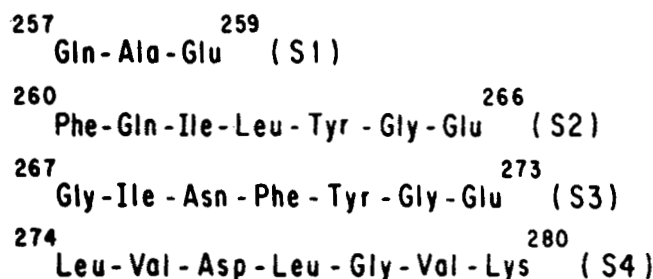


FIG. 6. Sequence of peptides resulting from *S. aureus* V8 protease digestion of *recA* tryptic peptide T31. Based on the known sequence of tryptic peptide T31 the sequences of the four peptides expected from *S. aureus* V8 protease digestion of T31 are presented. This prediction was confirmed by HPLC purification and amino acid compositional analysis of the *S. aureus* V8 peptides derived from T31 (see Fig. 7 and Table III).

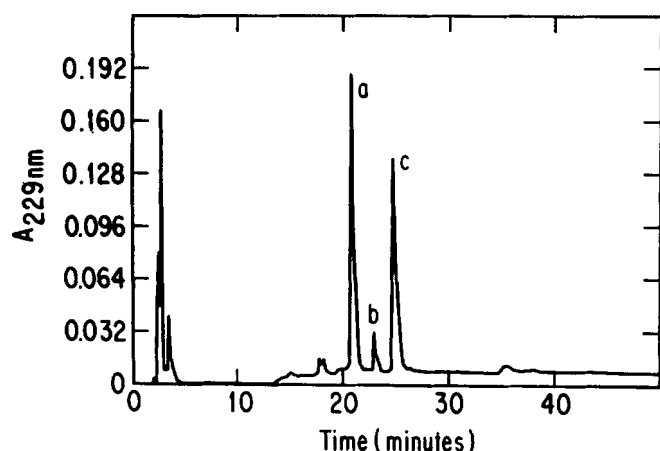


FIG. 7. C-4 HPLC purification of peptides resulting from *S. aureus* V8 protease digestion of underivatized T31. Peptides resulting from *S. aureus* V8 protease digestion of 6 nmol of underivatized T31 were separated on a Vydac C-4 reverse-phase column eluted at a flow rate of 1.0 ml/min. The profile was developed with a linear gradient of solvent B (70% CH₃CN) into solvent A (20 mM NaH₂PO₄/Na₂HPO₄, pH 6.8), 0–10 min (0–25% B), 10–110 min (25–45% B). Fractions were collected every 1.0 min. All peaks were lyophilized separately. Purification of underivatized T31 is described in the text.

on a C-4 column using gradient conditions identical to those in Fig. 7. The elution profile (Fig. 8) differed from that obtained with unmodified T31: no large peak c at 25 min (peptide S2, residues 260–266) was detected but two new peaks labeled *d'* and *e'* eluted considerably later in the gradient, at minutes 50–53 and 65–67, respectively. Peaks *d'* and *e'* accounted for more than 90% of the radioactivity loaded onto the column. Moreover, each peak absorbed strongly at 254 nm indicating attachment of the sulfonylbenzoyladenosine moiety. We determined the amino acid composition of each peak, *a'–e'*, and the results are presented in Table III. Peak *a'* was readily identified as peptide S3 containing residues 267–273. As indicated in Table III, all residues, including Tyr-271, were recovered in the expected molar yield. The composition of peak *b'* corresponded to peptide S4, which contains residues 274–280. Again, all amino acid residues were re-

covered quantitatively. A small peak eluting at 25 min (labeled *c'*) was analyzed but we could detect no values above background. A composition analysis of peak *d'*, which contained approximately 40% of the radioactivity, was consistent with that of peptide S2, residues 260–266, except that no tyrosine was recovered. Peak *e'*, which accounted for approximately 55% of the recovered ³H label, contained 3 Glx residues and had an amino acid composition consistent with a partial digestion product spanning residues 260–273. This product would be formed if cleavage at Glu-266 was blocked whereas cleavage occurred at both Glu-259 and Glu-273. This peptide gave the expected molar yields of all amino acids except for Tyr which showed a recovery of 1 mol/mol peptide rather than the expected value of 2. Based on the recovery of peptide material and radioactivity, we calculated a value of 1.3 mol of 5'-FSBA/mol of peptide for both peaks *d'* and *e'*. These results are consistent with Tyr-264 being the exclusive site of covalent linkage of 5'-FSBA to *recA* protein.

DISCUSSION

We have used the purine nucleotide analog 5'-FSBA to covalently modify *recA* protein within its ATP hydrolytic domain. At a constant protein concentration the rate of covalent modification shows a sigmoidal concentration dependence on 5'-FSBA. We were prevented from increasing the 5'-FSBA concentration beyond 2.5 mM due to the appearance of a variable amount of precipitate at higher levels of 5'-FSBA. Whether this was a result of protein or 5'-FSBA coming out of solution was not determined. However, this problem could not be remedied by lowering the protein concentration nor could it be prevented by increasing the amount of dimethylformamide in the reaction mixture in an effort to maintain the solubility of 5'-FSBA. Increasing the concentration of dimethylformamide above 3.5%, which was typically used in these experiments, led to inactivation of the *recA* protein ATPase activity. This dependence of the rate of labeling of *recA* protein on the concentration of 5'-FSBA suggests that it is characterized by positive cooperativity. Steady-state kinetic analysis also indicates positive cooperativity for hydrolysis of ATP by *recA* protein (24).

Covalent modification of *recA* protein with FSBA leads to irreversible inactivation of the enzyme's ATPase activity. The

TABLE III
Amino acid composition of *S. aureus* V8 protease peptides resulting from digestion of unmodified T31 and 5'-FSBA-modified T31

Lanes a–c are the amino acid compositions of the corresponding peaks in Fig. 7. Lanes *a'–e'* are the amino acid compositions of the corresponding peaks in Fig. 8. Numbers in parentheses are expected values determined from the known protein sequence (20). All experimentally determined values have been normalized based on the known composition of the peptides.

	a	b	c	a'	b'	d'	e'
Aspartic acid	1.0 (1)	1.0 (1)		1.0 (1)	1.1 (1)		1.0 (1)
Threonine							
Serine							
Glutamic acid	1.2 (1)		2.0 (2)	1.1 (1)		2.2 (2)	2.8 (3)
Proline							
Glycine	2.0 (2)	1.2 (1)	1.0 (1)	2.1 (2)	1.0 (1)	1.2 (1)	2.9 (3)
Alanine							
Valine		1.9 (2)			2.0 (1)		
Methionine							
Isoleucine	0.9 (1)		1.0 (1)	1.0 (1)		0.9 (1)	1.9 (2)
Leucine		1.7 (2)	1.0 (1)		2.0 (2)	1.0 (1)	1.1 (1)
Tyrosine	0.9 (1)		0.9 (1)	0.9 (1)		(1)	0.8 (2)
Phenylalanine	0.9 (1)		1.1 (1)	0.9 (1)		0.8 (1)	2.1 (2)
Histidine							
Lysine		1.0 (1)					
Arginine							
CM-cysteine							

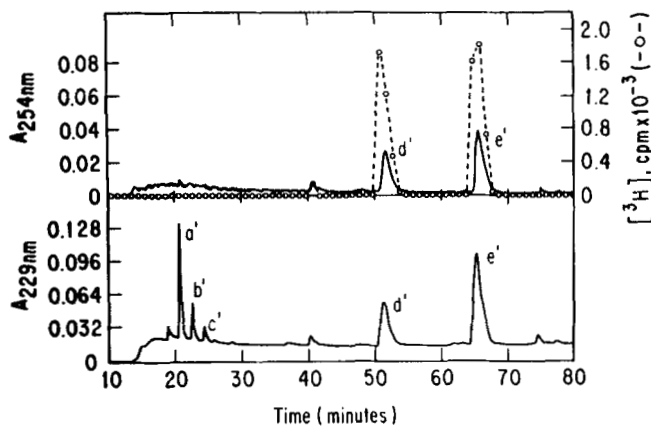


FIG. 8. C-4 HPLC purification of peptides resulting from *S. aureus* V8 protease digestion of 5'-FSBA-modified T31. Peptides resulting from *S. aureus* V8 protease digestion of approximately 10 nmol of 5'-FSBA-modified T31 were separated on a Vydac C-4 reverse-phase column eluted at a flow rate of 1.0 ml/min. The profile was developed as described in the legend to Fig. 7. Aliquots (40 μ l) of all fractions were assayed for ^3H counts/min. All peaks were lyophilized separately.

degree of inactivation is greater than would be expected for a given amount of modification. Extrapolation of the curve in Fig. 3 suggests that 100% inactivation of the *recA* protein ATPase activity is achieved when 25% of the enzyme monomers have been modified. With 5'-FSBA, we are able to modify *recA* protein almost quantitatively, i.e. 1 mol of 5'-FSBA/mol of protein. In other studies using the photoaffinity probe N_3 -ATP we reached a limit of only 0.2 mol of N_3 -ATP/mol of *recA* protein. In order to explain these results, we proposed that the active form of *recA* protein is a tetramer. Furthermore, we suggest that covalent modification of one subunit of the tetramer by 5'-FSBA or N_3 -ATP inactivates the other subunits with respect to ATP binding and hydrolysis. Indeed, there is evidence that mixing of active wild-type subunits and inactive mutant *recA* protein subunits can occur leading to inactivation of *recA* protein function (25, 26). However, as suggested by the cooperative 5'-FSBA labeling result, modification of one subunit leads to an enhanced rate of 5'-FSBA labeling at the others. Our kinetic and competition experiments indicate that unmodified *recA* protein has a low affinity for 5'-FSBA relative to ATP. The enhanced rate of 5'-FSBA labeling after the first analog molecule is attached may result from a conformational change in the ATP-binding sites that favors 5'-FSBA binding and reduces the ATP binding affinity. This model also explains the limited labeling by N_3 -ATP which is structurally very similar to ATP. After covalent attachment of N_3 -ATP at the first site, binding of the analog is blocked at the other sites in the tetramer. We would, therefore, expect a limit of 25% labeling with N_3 -ATP. This is, in fact, close to the upper limit of labeling that we have observed with N_3 -ATP (9).

That labeling with 5'-FSBA is occurring specifically within the ATP hydrolytic site of *recA* protein is supported by the fact that it is effectively competed by ATP and by ADP which is a competitive inhibitor of the *recA* protein ATPase activity. Additionally, UTP, which is a substrate for the *recA* protein nucleoside triphosphatase activity, and GTP and ATP γ S, which are both competitive inhibitors of this activity, significantly reduce the rate and extent of 5'-FSBA labeling.³ In contrast, adenosine, which is not an inhibitor of the ATPase activity, has no effect on 5'-FSBA labeling. These results

argue that 5'-FSBA recognizes and covalently binds to the ATP hydrolytic site of *recA* protein.

Using a two-step HPLC purification we isolated a single labeled peptide from a tryptic digest of *recA* protein that had been covalently modified by [^3H]5'-FSBA. This peptide was identified as T31, spanning residues 257–280. This result was unexpected since this same peptide had been identified as containing the unique site of labeling with the photoaffinity probe N_3 -ATP which covalently modifies nearby amino acids via the nitrene-free radical at the C-8 position of the adenine ring. Direct sequence analysis of peptide T31 labeled with either N_3 -ATP or 5'-FSBA was precluded by the presence of a blocked NH_2 -terminal glutamine residue. We, therefore, performed a secondary proteolytic digest on the labeled peptide using *S. aureus* V8 protease. HPLC purification of the resulting fragments gave rise to 2 labeled peptides, one of which (peak d', Fig. 7) was identified as *S. aureus* V8 peptide S2, residues 260–266, and the other (peak e', Fig. 7) being identified as a peptide spanning residues 260–273 resulting from partial proteolysis of the labeled tryptic peptide T31. As can be seen from a comparison of the HPLC profiles in Figs. 6 and 7, the attachment of 5'-FSBA to peptide S2 shifts its elution time dramatically. Unmodified S2 eluted at 25 min (peak c, Fig. 6) whereas, using identical gradient conditions 5'-FSBA-modified S2 did not elute until minutes 50–53 (peak d', Fig. 7). A significant retardation of the larger T31 peptide was also observed after 5'-FSBA labeling (Fig. 4). The amino acid composition of 5'-FSBA-labeled S2 is completely consistent with the site of FSBA attachment being located at Tyr-264. We recovered the expected molar amounts of all amino acids contained within S2 with the exception of tyrosine, for which no amount above background was detected. Amino acid analysis of the unmodified S2 resulted in the predicted molar yields of all residues including tyrosine. We have also shown that the recovery of tyrosine in *S. aureus* V8 peptide S3 is the expected 1 mol/mol peptide whether S3 is derived from the unmodified or 5'-FSBA-modified tryptic peptide T31.

The presence of peak e' in Fig. 7 further supports the localization of the 5'-FSBA at Tyr-264. This peak results from incomplete *S. aureus* V8 protease digestion at Glu-266. The amino acid composition reveals that this labeled peptide contains only 1 Tyr rather than the expected value of 2. Other amino acids were recovered in the expected yield based on the known peptide composition. Since Tyr-271 is unmodified in peptide S3 we conclude that in this partial digestion product Tyr-264 is again the site of 5'-FSBA attachment. It seems reasonable that the attachment of the large sulfonylbenzoyl-adenosine moiety at Tyr-264 would sterically impede and slow the rate of cleavage by *S. aureus* V8 protease 2 residues away at Glu-266.

Recently, we have demonstrated that covalent modification of *recA* protein with the photoaffinity ATP analog N_3 -ATP results in covalent attachment of the probe to the same tryptic peptide (T31) that is modified by FSBA. In the accompanying paper (23) we demonstrate that N_3 -ATP attaches to the same residue, Tyr-264, as 5'-FSBA and, together with these results discuss a likely configuration for adenine nucleotide binding to *recA* protein, and its position relative to Tyr-264.

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