lac Repressor Cysteine-140 Reacts Selectively with a Fluorescent Probe Bound to the Core–Headpiece Interface†

J. Michael Schneider,‡ Christine I. Barrett,§ and Sheldon S. York*

ABSTRACT: The fluorescent probe *N*-[[(iodoacetyl)amino]-ethyl]-5-naphthylamine-1-sulfonate (I-AEDANS) reacts selectively with Cys-140 of the *lac* repressor. The reasons for this selectivity were investigated. The ability of 8-anilino-1-naphthalenesulfonate and 5,5′-bis(8-anilino-1-naphthalenesulfonate) to bind noncovalently to the interface between the core and headpiece regions of the repressor suggested that I-AEDANS might also bind to this interface and then react intramolecularly with Cys-140 nearby. Two observations strongly support this model. (1) The selectivity for Cys-140 was lost when the headpiece regions were removed from the repressor. The rate of reaction with Cys-140 relative to Cys-107 in the repressor was 13.5 ± 1.4, from trypsin digestions of labeled repressor. This ratio decreased to 2.1 ± 1.0 for the core protein. (2) Iodoacetamide, which lacks the naphthylaminesulfonate portion of I-AEDANS, showed little selectivity for Cys-140 in either the repressor or the core. Nonreactive analogues of I-AEDANS did not alter the reaction of I-AEDANS with the repressor, presumably because they bound too weakly. Decreasing the ionic strength from 0.61 M to 56 mM decreased the selectivity of I-AEDANS for Cys-140 in the repressor, suggesting that I-AEDANS is not bound to the repressor by ionic interactions. Decreasing the pH from 8.5 to 7.5 increased the selectivity for Cys-140 only slightly. Fluorescent probes attached to Cys-140 appear to be ideally located to report motions of the headpieces, relative to the core, that attend DNA binding.

The *lac* repressor has been extensively studied as a model for understanding how a protein recognizes and binds tightly to a specific nucleotide sequence in DNA (Bourgeois & Pfahl, 1976; Miller & Reznikoff, 1978; Wu et al., 1978; Dunaway et al., 1980). Considerable information has been obtained by attaching probes to specific residues on the repressor which then report events affecting that site. Due to the unique reactivity of sulfhydryl groups, many of these probes have been selectively attached to cysteine residues. These probes have reported inducer binding (Friedman et al., 1976; Sams et al., 1977; Burgum & Matthews, 1978; Brown & Matthews, 1979b) and binding to both operator (Brown & Matthews, 1979b) and nonspecific DNA (Burgum & Matthews, 1978; Kelsey et al., 1979).

Each *lac* repressor subunit has three cysteine residues (Beyreuther et al., 1973). Several probes react selectively with Cys-140 and -107, leaving Cys-281 unlabeled. Of these, 2-(bromoacetamido)-4-nitrophenol (Yang et al., 1977) and *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonate (I-AEDANS)[1,2] (Kelsey et al., 1979) are especially selective for Cys-140. The AEDANS probe, attached to Cys-140, has been used to follow rapid conformational changes within the repressor as it binds to nonspecific DNA (Kelsey et al., 1979). In order to fully interpret the conformational changes reported by probes attached to Cys-140, it is essential to know where this residue is located within the three-dimensional structure of the protein.

Limited tryptic digestion selectively cleaves the repressor following Lys-59, producing a tetrameric core region (residues 60–360) and monomeric headpieces (residues 1–59) (Geisler & Weber, 1977). The headpiece regions are flexibly attached to the core within the native repressor (Buck et al., 1978; Wade-Jardetzky et al., 1979; Jarema et al., 1981). Once separated by tryptic digestion, these two regions appear to maintain their native structure. The repressor has an elongated shape, with two headpieces located at each end (Pilz et al., 1980; Charlier et al., 1980). The studies reported here indicate that Cys-140 is located at or near the interface between the core and headpiece regions of the repressor.

Materials and Methods

Isolation of Repressor, Cores, and Headpieces. The *lac* repressor was isolated from either *Escherichia coli* CSH 46 or *E. coli* pHIQ3. Growth of the CSH 46 strain has been described previously (Worah et al., 1978). The pHIQ3 strain, obtained from J. Sadler (Hare & Sadler, 1978), carries the i^q gene on the pMB9 plasmid. The bacteria were grown in the presence of 10 μg/mL tetracycline as described by Hare & Sadler (1978), and the cells were lysed by four freeze–thaw cycles in the presence of 0.5 mg/mL lysozyme. Repressor was purified by the method of Rosenberg et al. (1977) with modifications that have been described (Worah et al., 1978). Yields of purified *lac* repressor from 100 g of cell paste ranged from 50 to 100 mg. The purity of the repressor, determined from $NaDodSO_4$–polyacrylamide gels (Miller, 1972), was typically 98%. The repressor's ability to bind to nonspecific DNA was determined by titrating AEDANS-labeled repressor with poly[d(A-T)] (Kelsey et al., 1979). End points of 13–15 base pairs per tetramer were seen in these titrations, demonstrating that the repressor was fully active in binding to nonspecific DNA. The operator binding activity of the repressor was assayed by a filter binding technique (Riggs et

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‡ Present address: Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO.

§ Present address: Gates Energy Products, Denver, CO.

[1] Abbreviations: I-AEDANS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonate; AEDANS, *N*-[(acetylamino)ethyl]-5-naphthylamine-1-sulfonate group; ANS, 8-anilino-1-naphthalenesulfonate; bis(ANS), 5,5′-bis(8-anilino-1-naphthalenesulfonate); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, disodium ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)-aminomethane.

[2] This compound was abbreviated I-AENS in Kelsey et al. (1979).

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al., 1970) using ^{3}H-pMB9 plasmid DNA carrying a single *lac* operator at the *Eco*RI site (Sadler et al., 1978). This plasmid was purified from a thymine-requiring strain, *Escherichia coli* HOE260, obtained from J. Sadler. Typical repressor preparations were approximately 40% active in binding *lac* operator.

Repressor cores were prepared by the method of Geisler & Weber (1977). The repressor was digested with 1% w/w TPCK–trypsin for 2 h at 25 °C in 1.0 M Tris-HCl, pH 7.5 at 25 °C, 30% glycerol, and 0.01 M 2-mercaptoethanol, and the digestion was stopped by adding a 5-fold excess by weight of soybean trypsin inhibitor. Cores were isolated by using a 1.5 × 70 cm Sephadex G-150 column equilibrated with 0.1 M NH_4HCO_3. These core preparations were shown to be >95% homogeneous, missing only the 59 amino-terminal residues, by $NaDodSO_4$–polyacrylamide gel electrophoresis. Repressor headpieces were prepared by the method of Geisler & Weber (1977) as described previously (Worah et al., 1978).

Many different preparations of repressor and core proteins were used in the experiments reported here, with no differences in behavior being noted among different preparations.

The concentrations of these proteins were determined from their absorbance at 280 nm by using the following ϵ_{280} values: *lac* repressor subunit, 22 125 M^{-1} cm^{-1} (Huston et al., 1974); *lac* core subunit, 18 240 M^{-1} cm^{-1} (Huston et al., 1974); *lac* repressor headpiece, 5280 M^{-1} cm^{-1} (Worah et al., 1978).

Other Materials. 5,5′-Bis(8-anilino-1-naphthalenesulfonate) [bis(ANS)] (Molecular Probes and Regis), 5-(dimethylamino)-1-naphthalenesulfonic acid (Eastman), I-AEDANS (Aldrich), and iodoacetamide (Sigma) were used without further purification. 5-Amino-1-naphthalenesulfonic acid (Pfaltz and Bauer) was recrystallized 5 times from water. *N*-[(Acetylamino)ethyl]-5-naphthylamine-1-sulfonic acid was prepared by the method of Hudson & Weber (1973). TPCK–trypsin and soybean trypsin inhibitor were obtained from Worthington. Iodo[1-^{14}C]acetamide with a specific activity of approximately 23 Ci/mol was obtained from New England Nuclear.

Labeling Repressor and Cores. Both the repressor and core proteins were labeled with I-AEDANS (usually 8.4 mM), in the dark at 4 °C, as described previously (Kelsey et al., 1979). At the appropriate time, the protein was separated from excess reagent by gel filtration through Sephadex G-25 and then dialyzed for 24 h against standard buffer, with 0.1 mM dithiothreitol added. This standard buffer, used for most labeling experiments, contained 0.2 M Tris-HCl, pH 8.0 at 25 °C, 0.5 M KCl, and 0.1 mM EDTA. In experiments where the pH was varied, these concentrations were held constant as the composition of the Tris-HCl buffer was adjusted to give pH 7.0, 7.5, or 8.5 at 25 °C. Labeling at low ionic strength (56 mM) was carried out in 0.1 M Tris-HCl, pH 8.0 at 25 °C, and 0.1 mM EDTA. All buffer solutions were bubbled with N_2 before use to remove O_2. The concentration of AEDANS-labeled repressor or core and the extent of AEDANS labeling were determined from the absorbance at 280 and 340 nm by using the following values: *lac* repressor subunit, ϵ_{280} = 22 125 M^{-1} cm^{-1} (Huston et al., 1974), ϵ_{340} = 105 M^{-1} cm^{-1} (Kelsey et al., 1979); *lac* core subunit, ϵ_{280} = 18 240 M^{-1} cm^{-1} (Huston et al., 1974), ϵ_{340} = 87 M^{-1} cm^{-1}; AEDANS, ϵ_{280} = 1260 M^{-1} cm^{-1} (Kelsey et al., 1979), ϵ_{340} = 6850 M^{-1} cm^{-1} (Hudson & Weber, 1973).

The repressor and core proteins were labeled with 8.0 mM iodoacetamide in standard buffer at 4 °C in the dark. At the appropriate time, the reaction was terminated with 2-mercaptoethanol and the excess iodoacetamide removed by dialysis. Again, all buffers were bubbled with N_2 prior to use. The number of sulfhydryl groups that reacted with iodoacetamide was determined with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Habeeb, 1972).

Kinetic data giving the number of labels incorporated as a function of time were analyzed as one or the sum of two exponential terms by using a Levenberg–Marquardt routine (ZXSSQ), obtained from the International Mathematical and Statistical Libraries, Inc. (IMSL), for solving nonlinear least-squares problems. The confidence that two terms fit the data better than one term was judged according to the significance test employed by Laiken & Printz (1970) using the *R* factor tables given by Hamilton (1965).

Identification of Labeled Cysteine Residues. The procedure for identifying cysteine residues which reacted with I-AEDANS has been described (Kelsey et al., 1979). Briefly, AEDANS-labeled protein was denatured in 8 M urea, reacted with iodo[^{14}C]acetamide to label unreacted cysteine residues, dialyzed into 0.1 M NH_4HCO_3 and 2 M urea, and digested with TPCK–trypsin. The tryptic peptides were then separated on a Sephadex G-50 superfine column (1 × 50 cm) equilibrated with 0.1 M NH_4HCO_3 and 2% $NaDodSO_4$ at 25 °C. Thermostating this column improved the reproducibility of elution profiles.

In order to identify cysteine residues which reacted with iodoacetamide, the protein was reacted with 8.0 mM iodo-[^{14}C]acetamide at a specific activity of 2.3 Ci/mol. The ethanol present in the iodo[^{14}C]acetamide solution was removed by evaporation before the protein was added. Prior to TPCK–trypsin digestion, the ^{14}C-labeled protein was denatured in 8 M urea and the unreacted sulfhydryl groups were labeled by reaction with 9 mM iodoacetamide for 30 min at 32 °C.

Reactions with 2-Mercaptoethanol. The reaction of I-AEDANS and iodoacetamide with 2-mercaptoethanol was monitored both in an aqueous medium and in a medium containing an 80:20 (v/v) ethanol:H_2O mixture. The aqueous solution contained 0.2 M Tris-HCl, pH 8.3 at 25 °C, 0.5 M KCl, and 0.1 mM EDTA while the ethanol solution contained 0.2 M Tris-HCl, pH 8.3 at 25 °C, and 0.1 mM EDTA. Reactions were carried out at 4 °C in the dark. The initial concentration of both reactants was 0.01 M, and the disappearance of sulfhydryl groups was determined with DTNB (Habeeb, 1972).

Fluorescence Measurements. Fluorescence measurements were made with a Perkin-Elmer MPF-3 fluorometer using a 4-mm path-length cuvette at 25 °C. Studies with bis(ANS) added to repressor, cores, or headpieces were done in 0.1 M Tris-HCl, pH 8.0 at 25 °C, and 0.3 mM dithiothreitol, by using excitation and emission wavelengths of 398 and 486 nm, respectively. The fluorescence intensities were corrected for contributions from unbound bis(ANS) and for inner filter effects as described previously (York et al., 1978).

Results and Discussion

Noncovalent Probes Bind at the Core–Headpiece Interface. The fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS) binds to the *lac* repressor with a significant enhancement in fluorescence intensity (Worah et al., 1978). However, when ANS was added to the core protein or headpiece regions separately, the fluorescence enhancement was greatly diminished. These results were interpreted to mean that ANS binds at the interface of the core and headpiece regions of the repressor. Bis(ANS) behaves in a similar manner. When the repressor or core proteins, each 5.0 × 10^{-7} M in tetramers, were titrated with bis(ANS) from 2.0 × 10^{-6} to 2.1 × 10^{-5} M, the fluorescence enhancement obtained with cores was only 11% that obtained with the repressor. When 2.1 × 10^{-6} M

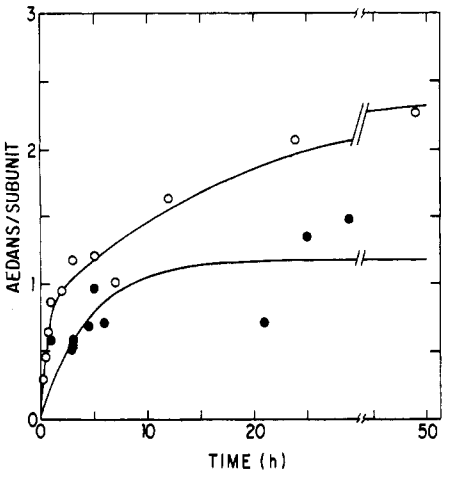

FIGURE 1: Labeling of repressor (O) and cores (●) with I-AEDANS. Repressor (4.5 μM tetramers) or cores (4.8–9.2 μM tetramers) were reacted with 8.4 mM I-AEDANS in standard buffer at 4 °C. The solid curves are best fits of these data. Labeling of repressor was best fit by the sum of two exponential processes with a confidence >99.5%. Labeling of cores was best fit by a single exponential process, which was forced to begin at zero. Rate constants derived from these data are given in the text.

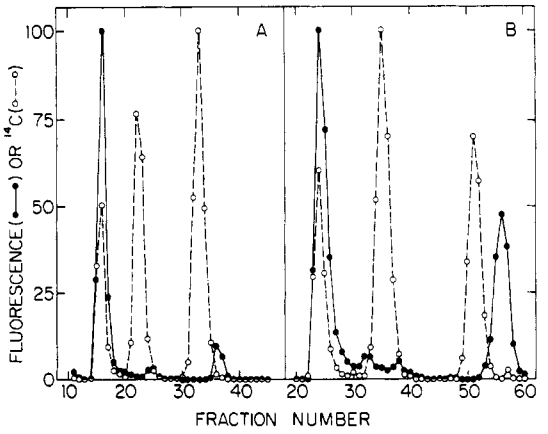

FIGURE 2: Separation of tryptic peptides from AEDANS-labeled repressor (A) and cores (B). The conditions of labeling were the same as in Figure 1. The fluorescence intensity of AEDANS-containing peptides (●) was monitored with excitation and emission wavelengths of 340 and 475 nm, respectively. The position of the sulfhydryl-containing peptides is marked with ^{14}C (O). Both fluorescence intensities and ^{14}C cpm are plotted on relative scales. (A) Digestion begun with 1.9 mg of repressor with 0.7 AEDANS label per subunit. Peak cpm (fraction 33) was 2398. (B) Digestion begun with 0.9 mg of cores with 0.7 AEDANS label per subunit. Peak cpm (Fraction 35) was 586. Background levels have been subtracted from both the fluorescence intensities and the cpm.

bis(ANS) was added to the repressor or headpieces at a monomer concentration of 2.0×10^{-5} M, the fluorescence enhancement obtained with headpieces was only 6% that obtained with the repressor.

The headpiece and core regions of the repressor form independent structural domains which are flexibly attached to each other (Buck et al., 1978; Wade-Jardetzky et al., 1979; Jarema et al., 1981). The headpieces retain their ability to bind correctly to the operator (Ogata & Gilbert, 1979) while the cores retain normal inducer binding (Files & Weber, 1976). Since these two regions of the repressor appear to retain their native structures when separated, and yet neither region alone can bind ANS or bis(ANS) normally, this implies that the binding site(s) for these fluorescent probes are created by the joining of these two regions, at the core–headpiece interface. Equilibrium dialysis experiments (Lawson, 1981) have shown that approximately four bis(ANS) molecules bind per repressor subunit with an average dissociation constant of 20 μM at 4 °C in the low (56 mM) ionic strength buffer. Since both ANS (Stryer, 1965) and bis(ANS) (Rosen & Weber, 1969) bind preferentially to nonpolar sites, all of these results taken together indicate that the core–headpiece interface is a relatively large nonpolar region, capable of binding several bis(ANS) molecules with similar affinities.

I-AEDANS reacts selectively with Cys-140 of the *lac* repressor (Kelsey et al., 1979). Since it is structurally similar to ANS and bis(ANS), perhaps it also binds at the core–headpiece interface and then reacts intramolecularly with a nearby cysteine residue, namely, Cys-140. Two direct consequences of this hypothesis can be readily tested: (1) removal of the headpiece regions should reduce the selectivity of I-AEDANS for Cys-140, and (2) iodoacetamide should not be selective for Cys-140.

Removing Headpieces Alters the Selectivity of I-AEDANS. The number of AEDANS labels incorporated per repressor or core subunit as a function of reaction time is shown in Figure 1. The reaction with the repressor is best fit by the sum of two exponential processes whereby 0.84 and 1.64 labels per subunit are introduced with pseudo-first-order rate constants of 1.79 and 0.050 h^{-1}, respectively. The reaction with the core is best fit by a single exponential process whereby 1.17 labels per subunit are introduced with a pseudo-first-order rate constant of 0.22 h^{-1}. These correspond to bimolecular rate constants of 5.9×10^{-2} and 1.7×10^{-3} M^{-1} s^{-1} for the repressor and 7.3×10^{-3} M^{-1} s^{-1} for the core. Figure 1 shows that a single residue in the repressor reacts rapidly with I-AEDANS, compared with the core.

Table I: Extent of Labeling at Cys-140 and -107

protein	I-AEDANS[a]	iodoacetamide[b]
repressor Cys-140	83	63
repressor Cys-107	9	26
core Cys-140	58	66
core Cys-107	32	23

[a] Percent of the total fluorescence intensity eluting from the columns in Figure 2. [b] Percent of total counts eluting from the columns in Figure 4.

The number of sulfhydryl groups that have reacted can be assessed with DTNB (Habeeb, 1972). The reaction of I-AEDANS with repressor has been shown previously to be primarily with cysteine residues (Kelsey et al., 1979). Analysis of core protein, labeled to different extents with AEDANS, has shown that more than 90% of the label has been introduced at cysteine residues.

The results of tryptic digestions to determine the selectivity of I-AEDANS labeling are shown in Figure 2. Tryptic digestion produces three cysteine-containing peptides that elute in the order Cys-140 (M_r 5361), Cys-281 (M_r 2860), and finally Cys-107 (M_r 693) (Kelsey et al., 1979). The Cys-107 peptide is delayed by the AEDANS label, presumably due to adsorption effects. Where the cysteine has been labeled first with AEDANS, the magnitude of the ^{14}C peak is reduced.

The results obtained with native repressor are shown in Figure 2A. The Cys-140 peptide accounts for 83% of the total fluorescence eluting from the column, while only 9% resides on the Cys-107 peptide (Table I). A higher percentage of the AEDANS label resides on Cys-140 than reported previously (Kelsey et al., 1979). TPCK–trypsin was used in this study while trypsin was used earlier. Apparently, there is enough contaminating chymotrypsin in trypsin to further degrade the Cys-140 peptide. The results obtained with the

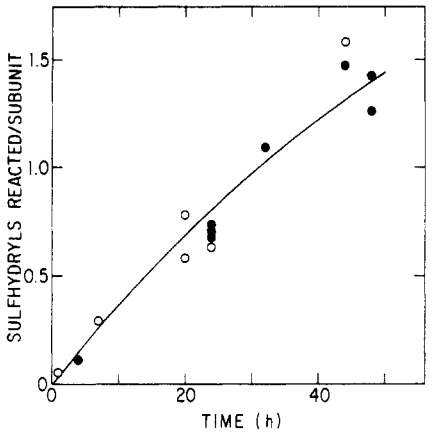

FIGURE 3: Labeling of repressor (O) and cores (●) with iodoacetamide. Repressor (5.6–16.7 μM tetramers) or cores (5.2–10.2 μM tetramers) were reacted with 8.0 mM iodoacetamide in standard buffer at 4 °C. The labeling of both repressor and cores was best fit by a single exponential process (solid curve), which was forced to begin at zero. The rate constant derived from these data is given in the text.

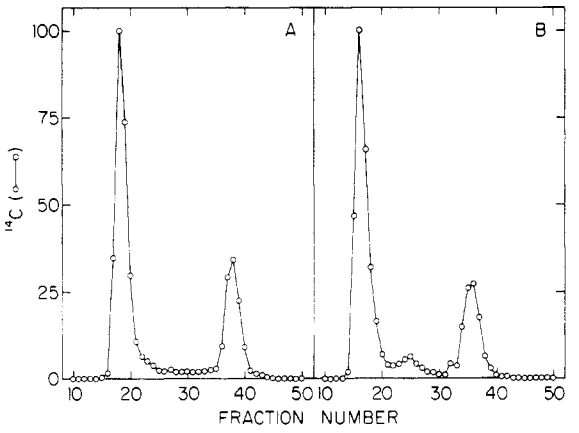

FIGURE 4: Separation of tryptic peptides from repressor (A) and cores (B) labeled with iodo[^{14}C]acetamide. Labeling proceeded for 24 h under the conditions given in Figure 3. ^{14}C cpm are plotted on a relative scale, after subtracting background levels. (A) Digestion begun with 1.1 mg of repressor with 0.5 sulfhydryl per subunit labeled. Peak cpm (fraction 18) was 10 722. (B) Digestion begun with 1.2 mg of cores with 0.6 sulfhydryl per subunit labeled. Peak cpm (fraction 16) was 2238.

core protein are shown in Figure 2B and tabulated in Table I. The selectivity which I-AEDANS shows for Cys-140 in the native repressor is significantly decreased by removal of the headpiece regions.

Iodoacetamide Is Not Selective for Cys-140. Figure 3 shows the number of sulfhydryl groups which have reacted as a function of time as iodoacetamide reacts with the repressor or core. The data for both repressor and core are best fit by a single exponential process whereby 3.0 sulfhydryls react with a pseudo-first-order rate constant of 0.013 h^{-1}. This corresponds to a bimolecular rate constant of 4.5×10^{-4} M^{-1} s^{-1}. Iodoacetamide reacts more slowly with the repressor and core than does I-AEDANS.

The results of tryptic digestions to determine the selectivity of iodoacetamide labeling are shown in Figure 4. Only two peaks containing radioactivity were observed, in the positions expected for the peptides containing Cys-140 and -107. The percent of total counts eluting from the column in the positions of the Cys-140 and -107 peptides is shown in Table I. Iodoacetamide shows little selectivity for Cys-140 over Cys-107 but does not react with Cys-281.

Two additional observations support the conclusion, drawn from the positions of the labeled peptides in Figure 4, that

Table II: Rate of Labeling of Cys-140 Relative to Cys-107 [k(Cys-140)/k(Cys-107)][a]

protein	I-AEDANS	iodoacetamide
repressor	13.5 ± 1.4	3.0 ± 0.3
core	2.1 ± 1.0	3.5 ± 0.4

[a] Calculated from tryptic digestion data.

iodoacetamide reacts primarily with cysteine residues in the repressor and core proteins. The number of iodoacetamide labels reacted with the core protein, as determined by the amount of ^{14}C incorporated, was compared with the number of sulfhydryl groups that had reacted, as determined with DTNB. This revealed that at least 85% of the iodoacetamide had reacted with sulhydryl groups. In reactions where the ethanol was not removed from the iodo[^{14}C]acetamide, the labeling solution contained as much as 18% (v/v) ethanol. At this concentration of ethanol, much of the repressor or core protein denatured and precipitated during the 24-h labeling procedure. When this precipitated protein was digested with trypsin and the tryptic peptides were separated, three radioactive peaks of similar size were observed, in the positions expected for the peptides containing Cys-140, -281, and -107. While these repressor and core proteins were sufficiently denatured to expose Cys-281 for reaction, apparently no residues other than cysteines reacted with iodoacetamide.

The extents of labeling at Cys-140 and -107 seen in these tryptic digests (Table I) provide another means of determining the relative reactivities of these residues. Assuming that these residues react independently in a pseudo-first-order manner, then the ratio of the pseudo-first-order rate constants for reaction at Cys-140 and -107 is related to the unreacted fraction of these residues by the following relationship:

$$\frac{k(\text{Cys-140})}{k(\text{Cys-107})} = \frac{\ln (\text{fraction of Cys-140 unreacted})}{\ln (\text{fraction of Cys-107 unreacted})}$$

Table II gives average values for these ratios, calculated from the data presented in Table I and additional digestion data. Again, it is readily apparent that I-AEDANS reacts selectively with Cys-140 in the native repressor, but not in the core, and that iodoacetamide shows little selectivity for Cys-140 in either the repressor or the core.

The ratio of the rate constants for the reaction of I-AEDANS with Cys-140 and -107 in the native repressor determined in this manner, 13.5 ± 1.4 (Table II), can be compared with the ratio determined from computer fits of data such as shown in Figure 1, 31 ± 8. This latter value probably overestimates the selectivity of I-AEDANS for Cys-140. The computer fit averages the reaction of Cys-107 with that of other residues which react more slowly (Kelsey et al., 1979), thereby underestimating the rate of Cys-107 reaction.

The simplest explanation of these data is that I-AEDANS binds to the interface of the core and headpiece regions of the repressor and then reacts intramolecularly with Cys-140 which happens to be nearby. This enhances the rate at which Cys-140 reacts relative to Cys-107. When the headpiece regions are removed, this binding site for I-AEDANS is destroyed, and the selectivity for Cys-140 is lost. Similarly, when the naphthylamine sulfonate portion of the label is removed, binding to this site is lost, and Cys-140 and -107 again react at similar rates. The manner in which ANS and bis(ANS) bind suggests that the binding site is located at the core–headpiece interface and not within the headpiece region itself. In an alternate model, the binding of I-AEDANS to the interface could promote a conformational change within the repressor, enhancing the reactivity of Cys-140 with a second

molecule of I-AEDANS at some distance from the interface.

Search for Competitive Inhibitors. The first model predicts that molecules similar to I-AEDANS but lacking the reactive iodoacetamide group should compete with I-AEDANS for binding to the repressor and decrease the reactivity of, and selectivity for, Cys-140. The second model predicts that these molecules, like I-AEDANS, might well enhance the reactivity of Cys-140. The following compounds with structures similar to that of I-AEDANS have been tested for their ability to influence the incorporation of AEDANS labels within the repressor as a function of time: 5-amino-1-naphthalenesulfonic acid at 8×10^{-5} to 8×10^{-2} M, 5-(dimethylamino)-1-naphthalenesulfonic acid at 8×10^{-5} to 3×10^{-2} M, and *N*-[(acetylamino)ethyl]-5-naphthylamine-1-sulfonic acid at 4×10^{-4} to 5×10^{-2} M. None had any noticeable effect. Furthermore, 5-amino-1-naphthalenesulfonic acid at 2×10^{-2} and 8×10^{-2} M had no effect on the percent of AEDANS label attached to Cys-140 and -107 as determined from tryptic digestions.

The explanation for the inability of these compounds to influence the reaction of I-AEDANS with the repressor probably lies in their weak binding. This is suggested by an analysis of how tightly I-AEDANS itself would have to bind to the repressor to give the observed enhancement in the rate of reaction with Cys-140. If one assumes that I-AEDANS first binds to the repressor and then reacts intramolecularly with Cys-140 nearby, i.e., by the reaction scheme

$$\text{repressor} + \text{I-AEDANS} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{repressor–I-AEDANS}] \xrightarrow{k_2} \text{AEDANS–repressor}$$

then the observed first-order rate constant ($k_{\text{I-AEDANS}}$) is given by

$$k_{\text{I-AEDANS}} = \frac{k_2[\text{I-AEDANS}]}{[\text{I-AEDANS}] + K_D}$$

where $K_D = k_{-1}/k_1$ if $k_{-1} \gg k_2$. On the other hand, for iodoacetamide which presumably reacts bimolecularly, i.e., by the reaction scheme

$$\text{repressor} + \text{iodoacetamide} \xrightarrow{k_R} \text{labeled repressor}$$

the observed first-order rate constant (k_{IA}) is given by $k_{IA} = k_R$[iodoacetamide]. Since I-AEDANS and iodoacetamide have the same inherent reactivity for sulfhydryl groups (see below), the value of k_R, 4.5×10^{-4} M^{-1} s^{-1} (Figure 3), can be used to estimate the value of k_2. Assuming that the effective local concentration of I-AEDANS within the [repressor–I-AEDANS] complex is equivalent to 5 M, as often observed in simple organic reactions (Glazer et al., 1975), the value of k_2 is given by $k_2 = k_R$[5 M]. The value of $k_{\text{I-AEDANS}}$ from Figure 1 is 1.79 h^{-1}, giving $K_D = 3 \times 10^{-2}$ M. Another estimate of K_D can be made by assuming that I-AEDANS reacts with Cys-107 in a bimolecular fashion. The ratio of rate constants for reaction at Cys-140 and -107, 13.5 (Table II), then yields a value for K_D of 0.4 M. These K_D values are consistent with the low affinity the repressor has for ANS (York et al., 1978). If the compounds tested for their inhibitory properties had bound to the repressor with similar affinities, then one would not have observed any competition. Unfortunately, the limited solubilities of these compounds prohibited the use of higher concentrations.

The ability of bis(ANS) to influence the reaction of I-AEDANS with the repressor was also tested. Neither 0.1 mM bis(ANS) in the standard labeling buffer nor 1 mM bis(ANS) in the low ionic strength buffer had any significant effect on the rate at which I-AEDANS labeled the repressor. Given the average dissociation constant for bis(ANS), 20 μM in the low ionic strength buffer, an effect should have been observed if bis(ANS) and I-AEDANS bound to the same site. However, it seems quite plausible that I-AEDANS and bis(ANS) bind simultaneously to the core–headpiece interface. Since several molecules of bis(ANS) apparently bind to this interface, there appears to be ample room to accommodate I-AEDANS even in the presence of bis(ANS). Furthermore, because of its smaller size, I-AEDANS might bind to a site from which the larger bis(ANS) is excluded.

Since the compounds tested had no effect on the reaction between the repressor and I-AEDANS, they do not help distinguish between the two models presented earlier, thereby inviting additional models. For example, Cys-140 might be located at some distance from the core–headpiece interface in a nonpolar environment that favors its accessibility to I-AEDANS but not to iodoacetamide. Removal of the headpieces might then cause a conformational change in the core which lessens the reactivity of Cys-140 with I-AEDANS. Given the available data, models such as this cannot be ruled out. However, the model where I-AEDANS binds to the core–headpiece interface and then reacts intramolecularly with Cys-140 nearby accounts most simply for all of the data. The core–headpiece interface appears to be a large nonpolar region capable of binding I-AEDANS. Furthermore, this model directly accounts for the effects of removing headpieces, without having to invoke conformational changes within the core. It is unfortunate that the weak binding of I-AEDANS and similar compounds prevents a more direct test of this model by kinetic means (Glazer et al., 1975).

The inherent reactivity of I-AEDANS and iodoacetamide toward sulfhydryl groups is essentially the same, as judged by their reaction with 2-mercaptoethanol. These reactions were carried out both in an aqueous solution and in an 80:20 (v/v) ethanol:H_2O mixture, which corresponds more closely to the environment about Cys-140 in the repressor. This is based on the observation that the emission maximum of the AEDANS label on Cys-140 is 475 nm (Kelsey et al., 1979), close to the emission maximum observed in 80% (v/v) ethanol (Hudson & Weber, 1973). The bimolecular rate constants for I-AEDANS and iodoacetamide reacting with 2-mercaptoethanol in an aqueous solution were 1.1 and 1.2 M^{-1} s^{-1}, respectively. The corresponding rate constants in the ethanol solution were 0.046 and 0.033 M^{-1} s^{-1}, respectively.

Effect of pH and Ionic Strength on Selectivity of I-AEDANS. In attempts to find conditions where the selectivity of I-AEDANS for Cys-140 in the repressor would be enhanced, both the pH and ionic strength of the reaction media were varied. In reactions at pH values ranging from 7.0 to 8.5, measured at 25 °C, with the concentration of KCl held constant at 0.5 M to maintain the ionic strength in the range of 0.56–0.68 M, the ratio of k(Cys-140)/k(Cys-107) determined from tryptic digestion data did not vary appreciably. As the pH was decreased from 8.5 to 7.5, this ratio increased from 10.0 to 13.8. The reaction is somewhat more selective at lower pH, but the rate of reaction is also decreased, necessitating longer labeling times. For example, at pH 7.0, the rate of reaction was only one-fifth that at pH 8.0.

The reaction was also carried out in 0.1 M Tris-HCl, pH 8.0 at 25 °C, with an ionic strength of 56 mM. The selectivity for Cys-140 decreased, the ratio k(Cys-140)/k(Cys-107), determined from tryptic digestions, being only 7.1 ± 3.0. Furthermore, the rate of reaction with Cys-140 had decreased. This implies that the binding of I-AEDANS to the repressor

does not involve ionic interactions between the negatively charged sulfonate group and positively charged groups on the repressor. Lowering the ionic strength would have increased binding to the repressor and hence the selectivity for Cys-140 if ionic interactions were involved. Brown & Matthews (1979a), on the basis of the chemical reactivity of Cys-140 toward a variety of modification reagents, concluded that Cys-140 resides in an apolar region of the repressor's three-dimensional structure. The results presented here are consistent with this interpretation.

Conclusion. These studies suggest that I-AEDANS reacts selectively with Cys-140 in the native repressor because of an affinity labeling effect. By binding to a site at the interface of the core and headpiece regions of the repressor, it reacts intramolecularly with Cys-140 nearby. This is an excellent location from which to monitor the binding of the repressor to DNA. At least two (Geisler & Weber, 1976), and perhaps all four (Dunaway & Matthews, 1980), headpieces directly contact the operator. The headpieces are flexibly attached to the core (Buck et al., 1978; Wade-Jardetzky et al., 1979; Jarema et al., 1981). It is thought that this flexibility enables the headpieces to properly align themselves with the DNA. We previously showed that the AEDANS label attached to Cys-140 reports two distinct intramolecular conformational changes as the repressor binds to the nonspecific DNA, poly[d(A-T)] (Kelsey et al., 1979). This study suggests that these changes are motions of the headpieces, relative to the core, that attend DNA binding.

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Registry No. I-AEDANS, 36930-63-9; ANS, 82-76-8; bis(ANS), 63741-13-9; iodoacetamide, 144-48-9; cysteine, 52-90-4.

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