5-Hydroxytryptophan as a New Intrinsic Probe for Investigating Protein–DNA Interactions by Analytical Ultracentrifugation. Study of the Effect of DNA on Self-Assembly of the Bacteriophage λ *c*I Repressor†

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ABSTRACT: Pairwise cooperativity between proteins bound to DNA is believed to be important in governing the transcriptional regulation of numerous genes. However, the spectral overlap of normal proteins and DNA has blocked the study of these interactions by many physical methods. As shown recently by Ross et al. (in press), λ *c*I repressor spectrally enhanced by 5-hydroxytryptophan (5-OHTrp), expressed in vivo using an *Escherichia coli* tryptophan auxotroph, exhibits dimer formation and DNA binding properties identical with those of the wild-type repressor. Moreover, the 5-OHTrp provides a spectral signal that allows monitoring of the protein concentration without interference from DNA. In this article, the ability to selectively detect 5-OHTrp-labeled repressor during analytical ultracentrifugation is used to study the higher order assembly of repressor dimers in the absence and in the presence of operator DNA. Contrary to the expectation that tetramer might be the limiting oligomer, λ *c*I repressor undergoes a definite association to octamer. The relatively narrow concentration range over which the transition from predominantly dimer to predominantly octamer occurs makes it unlikely that significant levels of tetramer are formed in the absence of DNA. Moreover, mass measurements reveal that an O_R1 oligonucleotide binds to octameric repressor and does not dissociate it to tetramers. The use of the 5-OHTrp spectral enhancement opens a promising new avenue for the exploration of protein–protein and protein–nucleic acid interactions by analytical ultracentrifugation.

It is widely accepted that transcriptional control by λ *c*I repressor is dependent on pairwise cooperative interactions between repressor dimers bound to adjacent sites at the three-site operators, O_R and O_L (Ptashne, 1992). Cooperativity is believed to be mediated by protein–protein interactions between repressor dimers, which associate to form tetramers. To account for cooperativity in binding and in transcriptional regulation, this protein self-association mechanism requires that the tetramers be preferentially stabilized when repressor is bound to DNA. However, the ability to study the linkage between protein–protein interactions and protein–DNA interactions has been hampered by the lack of a good spectral means for distinguishing protein from DNA.

Recently, Ross and Senear and their co-workers (Ross et al., 1992) and Szabo and co-workers (Hogue et al., 1992) showed that 5-hydroxytryptophan[1] (5-OHTrp) has a strong absorbance signal at 310 nm, a wavelength where DNA absorption is negligible. λ *c*I expressed using an *Escherichia coli* tryptophan auxotroph grown in a medium containing 5-OHTrp produces λ *c*I in which greater than 95% of the tryptophan is replaced by 5-OHTrp (Ross et al., in press). This protein is indistinguishable from wild-type repressor with respect to both dimer formation and cooperative binding to specific operator DNA.

Contrary to the widely held expectation that tetramers would be a stable association complex, we find that in the absence of DNA, and over a protein concentration range between 4 and 70 μM, both wild-type and 5-OHTrp λ *c*I repressors reversibly associate to octamers, with tetramers forming only as an intermediate species in the polymerization pathway. A full analysis of the assembly scheme is beyond the scope of this article, but will be available later (D. F. Senear, J. B. A. Ross, and T. M. Laue, in preparation).

It has been recognized for some time that having a unique spectral signal simplifies the analysis and interpretation of sedimentation data from heteroassociating systems. We have found that the absorbance of 5-OHTrp-λ *c*I repressor at 310 nm permits the investigation of the higher order assembly of repressors when bound to operator DNA. This approach will prove extremely useful in determining the mechanisms involved in pairwise cooperativity in the λ system. Perhaps more importantly, it offers the strong possibility of a powerful and general new tool for studying protein–DNA and protein–protein interactions.

METHODS

Proteins and Operator DNA. Expression and isolation of wild-type λ *c*I and 5-OHTrp-λ *c*I were performed as described elsewhere (Ross et al., in press). Repressor was further purified by chromatography on a Superdex 75 HR 10/30 FPLC (Pharmacia, Inc.) column. Protein concentration was estimated from average 280-nm extinction coefficients for tryptophan (5500 M^{-1} cm^{-1}) and tyrosine (1200 M^{-1} cm^{-1})

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[1] Abbreviations: 5-OHTrp, 5-hydroxytryptophan; 5-OHTrp-λ *c*I, 5-hydroxytryptophan-λ *c*I, 5-hydroxytryptophan bacteriophage λ *c*I repressor; ss, single strand; ds, double strand; bp, base pair; FPLC, fast protein liquid chromatography.

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residues in a protein (Wetlaufer, 1962) and the 280-nm extinction coefficient of 5-OHTrp incorporated in a polypeptide (4500 M^{-1} cm^{-1}) (Ross et al., in press), assuming additivity of the absorbances. The monomer molecular weight of the 5-OHTrp-λ *c*I repressor is 26 240, based on its sequence (Sauer & Andregg, 1978).

Oligonucleotides (21 bp) containing the plus and minus single-strand (ss) sequences of the λ operator, O_R1, were purchased from Oligos, Etc. These were annealed to double-stranded (ds) DNA by heating an equimolar mixture in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) to 85 °C in a dry bath for 15 min and then transferring it to a 70 °C water bath. The sample was allowed to cool to room temperature at an initial rate of 4–5 °C/h. To separate the annealed ds DNA from residual ss DNA, the mixture was subjected to FPLC on a BioRad EconoPac Mono Q cartridge. The column was eluted with a 50-mL gradient from 0.0 to 1.0 M NaCl in TE buffer. The ds oligonucleotide eluted in a sharp peak at about 0.6 M NaCl. Small peaks and shoulders at both higher and lower NaCl concentrations indicated contamination by UV chromophores. We presumed these were oligonucleotides which were not fully deprotected during their synthesis. Peak fractions which were judged to be pure DNA, on the basis of their absorption spectra from 220 to 300 nm, were pooled. The size of the DNA and removal of residual ss material were confirmed by acrylamide gel electrophoresis using the Gibco/BRL 10-bp DNA ladder as markers. Oligonucleotide concentration was estimated by assuming an extinction coefficient of 20 L g^{-1} cm^{-1} at 260 nm (Maniatas, 1982).

Analytical Ultracentrifugation. Proteins were prepared for centrifugation by exhaustive dialysis against 10 mM Tris, pH 8.0, 200 mM KCl, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 0.1 mM dithiothreitol. O_R1 oligonucleotide was prepared by twice precipitating it with ethanol and redissolving it in this buffer. Oligonucleotide was added directly to the protein from a 26 μM solution to yield a 1.5-fold molar excess of oligonucleotide to total repressor concentration (as a dimer). High-speed sedimentation equilibrium experiments were conducted at 10 000 rpm, 23 °C, in a Beckman XL-A analytical ultracentrifuge using an absorbance optical system, a four-hole titanium rotor, six-channel, 12-mm-thick charcoal–epon centerpieces, and fused silica windows. Sedimentation data were acquired as an average of four absorbance measurements per radial position and at a nominal radial spacing of 0.001 cm. Absorbance spectra were obtained at 1-nm intervals at mid-cell radial positions using an average of four intensity measurements. Data were edited using REEDIT (kindly provided by David Yphantis) and analyzed using NONLIN (Johnson et al., 1981).

Conversion between reduced molecular weights, σ (Yphantis & Waugh, 1956), and molecular weights used a calculated buffer density of 1.04 g/mL (Laue et al., 1992), a partial specific volume ($\bar{\nu}$) of 0.736 mL/g for 5-OH-Trp-λ *c*I calculated from the amino acid composition of wild-type λ *c*I (Cohn & Edsall, 1943), and an assumed $\bar{\nu}$ of 0.55 mL/g for the O_R1 DNA oligonucleotide (Durschlag, 1986).

RESULTS AND DISCUSSION

Absorbance Spectra. The absorbance profiles for O_R1, wild-type λ *c*I, 5-OHTrp-λ *c*I, and a 1.5:1 mole ratio mixture of O_R1/5-OHTrp-λ *c*I are presented in Figure 1. Two features are noteworthy. First, the absorbance of the DNA alone covers the same spectral range as the wild-type protein and effectively masks the protein contribution over the entire range. For a mixture of protein and DNA, it is impossible to deconvolute

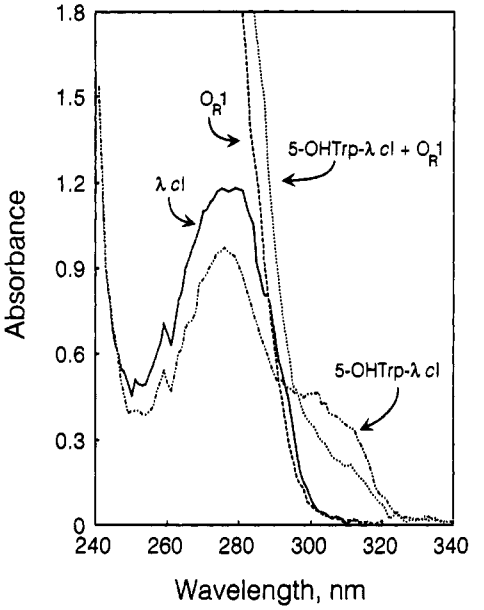

FIGURE 1: Absorption spectra of wild-type λ *c*I in the absence of DNA (—) and O_R1 (- - -) and 5-OHTrp-λ *c*I in the absence (- · -) and presence of O_R1 (···) in 20 mM Tris (pH 8), 200 mM KCl, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 0.01 mM dithiothreitol buffer, measured in the Beckman XLA ultracentrifuge.

a spectrum accurately into the two component spectra: either the DNA absorbance overwhelms the contribution from protein (i.e., wavelengths less than 280 nm) or the DNA absorbance is decreasing sharply (i.e., wavelengths greater than 280 nm), so that small errors in the total absorbance result in large errors in the calculation of the component concentrations. Second, 5-OHTrp-λ *c*I has a well-defined, broad absorbance shoulder between 300 and 320 nm where DNA absorbance approaches zero. Therefore, absorbance measurements made at 310 nm provide the concentration of 5-OHTrp-containing protein without interference from DNA. This permits the assessment of the state of oligomerization of 5-OHTrp-λ *c*I in the presence of DNA.

Sedimentation of 5-OHTrp-λ cI. 5-OHTrp-λ *c*I forms dimers (52 480 Da) with the same k_d value (~30 nM) as the wild-type repressor (Koblan & Ackers, 1991; Ross et al., 1992). Likewise, we find that these dimers form oligomers at concentrations exceeding 1 μM, with octamer (210 000 Da) being the limiting species for both wild-type λ *c*I (Senear et al., in preparation) and 5-OHTrp-λ *c*I (Table I). These results are consistent with those of Pirotta et al. (1970), who described a dimeric 50-kDa species undergoing mass-action assembly to forms in the range of 160–180 kDa. From this they inferred the existence of tetramers (~100 kDa), which are presumed to form the basis for pairwise cooperativity when bound to operator DNA (see Ptashne (1992)). Our results indicate that higher order assemblies should be taken into consideration in the interpretation of pairwise cooperativity. At the concentrations presented in Table I, the 5-OHTrp-λ *c*I is predominantly octameric in the absence of DNA. However, it should be noted that at the lower concentration (down to 4 μM as monomer) there is some dissociation as evidenced by the decrease in the whole cell average molecular weight (Table I). Data acquired at lower concentrations demonstrated that these molecules dissociate to dimers (data not shown), with a sharp gain in molecular weight occurring in the low micromolar range. In the equilibrium experiments presented here, the pressure head never exceeds a few atmospheres, both because of the relatively short solution column and because of the relatively low rotor speeds used. For octamer formation,

Table I: Molecular Weights of 5-OHTrp-λ *c*I with and without O_R1 Oligonucleotide

sample[a]	[protein][b] (μM)	σ[c] (cm^{-2})	M_r[d]	rms[e] (A_{310})
5-OHTrp-λ *c*I	38	2.26	217 000	0.009
	(7.5, 72)	(2.21, 2.30)	(213 000, 221 000)	
	11.5	2.01	193 000	0.003
	(3.8, 25)	(1.97, 2.05)	(189 000, 197 000)	
1.5:1 O_R1/ 5-OHTrp-λ *c*I	19	2.74	225 000–250 000[f]	0.005
	(1.0, 37)	(2.62, 2.85)		

[a] Experimental conditions are described in the Methods section. [b] Protein concentration (as monomer) loaded into the cell with meniscus and base concentrations indicated in parentheses. [c] Reduced molecular weight as defined by Yphantis and Waugh (1956). Values in parentheses show the 65% confidence interval (Johnson et al., 1981). [d] Molecular weight calculated from σ, assuming $\bar{v}$ = 0.736 mL/g for the repressor and $\bar{v}$ = 0.698 mL/g (i.e., 1:1 O_R1/5-OHTrp-λ *c*I dimer) in the presence of DNA. [e] Root mean square of the variance of the fit. [f] Uncertainty in the DNA binding stoichiometry leads to uncertainty in $\bar{v}$, so that conversion from σ to M_r is less assured. Therefore, the range of M_r includes either two or three O_R1 oligonucleotides per octamer. σ should be used for comparison with Table II.

Table II: Effect of O_R1 Binding Stoichiometries on the Predicted σ of Octamer Repressor

mol of O_R1 bound/octamer	expected M_r	predicted σ[a]
0	210 000	2.20
1	223 000	2.45
2	237 000	2.70
3	251 000	2.97
4	264 000	3.21

[a] σ was calculated for 10 000 rpm, taking into account the DNA contribution to the mass and $\bar{v}$ at each DNA:octamer stoichiometry.

the expected volume change in going from dimers to octamers is small, probably lying in the direction of dissociation, and using generous estimates for the magnitude of ΔV is not nearly sufficient to perturb the equilibrium. Moreover, cell average molecular weights determined at different rotor speeds (not shown) exhibited no systematic change, as would be expected if pressure did affect the equilibrium.

One obvious question is whether or not octameric repressor binds DNA. The absorbance of 5-OHTrp at 310 nm allows comparison of the state of oligomerization of 5-OHTrp-λ *c*I in the presence and absence of DNA. The protein concentration in the presence of O_R1 (19 μM, Table I) was selected to poise the equilibrium near the range over which significant octamer dissociation to dimer occurs. Thus, any tendency toward dissociation resulting from O_R1 binding should be readily apparent as a decrease in the reduced molecular weight (σ, which is directly proportional to the molecular weight (Yphantis & Waugh, 1956)). The σ of 5-OHTrp-λ *c*I repressor increases 20% in the presence of a 1.5-fold molar excess of O_R1 oligonucleotide over that found in the absence of DNA (Table I). This clearly demonstrates that DNA binding is not accompanied by dissociation of the octamers, as it must be if only smaller protein oligomers bind DNA. Rather, this result indicates that the octamer is competent to bind this oligonucleotide. Essentially identical results were obtained for two different preparations of protein and oligonucleotide.

To better appreciate the advantage provided by using the unique spectral properties of 5-OHTrp, it is instructive to determine the molecular weight of the mixture of protein and DNA using the concentration distributions determined at 280 nm. Using these data, the σ decreases to 1.69 ± 0.04, indicating a whole cell molecular weight average of 138 000. This decrease in σ reflects the contribution made by the excess DNA (13 600 Da) to the molecular weight average. This is confirmed by data acquired at 30 000 rpm, where the repressor octamer:oligonucleotide complexes are nearly pelleted (σ > 28) and the whole cell molecular weight average decreases to 14 000–15 000. It is important to note that it is impossible to properly interpret the 280-nm data without knowledge of the exact equilibrium association constants for all possible interactions, as well as accurate extinction coefficients and $\bar{v}$ for all species.

It is of interest to compare the measured σ's (Table I) with those predicted for 5-OHTrp-λ *c*I octamers binding varying amounts of O_R1 DNA (Table II). Using 310 nm as the observation window, it is clear that the expected σ for an octamer and that observed for 5-OHTrp-λ *c*I in the absence of DNA are very similar (Table I). The addition of oligonucleotide at a mole ratio of 1.5:1 operator:repressor dimer results in a 20% increase in σ, indicative of operator binding by the octamer. The exact stoichiometry of binding is not clear from these data, although comparison of the measured values of σ (Table I) with the predicted values (Table II) suggests that the stoichiometry is about 2–3. Why this is less than the expected stoichiometry of four oligonucleotides per octamer is not clear at this time, but it could result from nonsaturation of the potential binding sites, sample heterogeneity, errors in the assumed $\bar{v}$, or small amounts of octamer dissociation. The salient point is that, by using the 310-nm observation window, we are able to show that O_R1 oligonucleotide binds to the octamer and that binding does not drive the octamer to a less associated state.

The higher order assembly of repressor and the effect of DNA binding on this process bear directly on the mechanisms governing cooperativity both in repressor binding and in transcriptional regulation. Repressor dimers assemble to octamers over a rather narrow concentration range (Table I). This means that tetramers can be present only as an intermediate in the overall assembly pathway in free solution. Estimates of the fractional amount of tetramer must await a more complete analysis of the assembly process. However, it should be plain that the amount cannot be very great if dimer predominates at 1 μM (Koblan & Ackers, 1991; Ross et al., 1992) and octamer predominates at 40 μM (Table I). Furthermore, it is not reasonable to propose that binding of the repressor to DNA somehow alters the significance of octamer formation in free solution. The observation that binding of specific operator DNA does not dissociate octamers to tetramers (Table I) demonstrates that tetramer does not become a preferred oligomer when repressor dimer is bound to DNA. Indeed, these thermodynamic results dictate that octamer formation by the repressor must be included in any schemes describing the role of polymerization in cooperativity. There is no choice; the energy required to destabilize the octamer would have to be accounted for in any models that involve solely tetramer as the cooperative unit.

Previous results (Ross et al., 1992) and those presented here demonstrate that in vivo substitution of 5-OHTrp for tryptophan yields λ *c*I repressor that is spectroscopically distinct, but otherwise indistinguishiable, from wild-type repressor. Thus, 5-OHTrp appears to be a structurally benign substitute for tryptophan, suggesting that it may be useful as a more general probe for spectrally enhancing proteins. The present results demonstrate that spectroscopic detection of proteins containing 5-OHTrp, combined with analytical ultracentrifugation, allows quantitative assessment of homomeric protein polymerization in the presence of DNA. However, sedimentation of proteins selectively labeled with

5-OHTrp should prove to be extremely useful in the study of a wide range of protein–DNA and heteromeric protein–protein interactions. Mixing studies, such as those presented here, in which the associative state of one type of molecule can be determined in the presence of many other kinds will allow the dissection of complicated interactions in a way that previously was not possible.

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