The N Terminus of *Myxococcus xanthus* CarA Repressor Is an Autonomously Folding Domain That Mediates Physical and Functional Interactions with Both Operator DNA and Antirepressor Protein*

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Expression of the *Myxococcus xanthus* carB operon, which encodes the majority of the enzymes involved in light-induced carotenogenesis, is down-regulated in the dark by the CarA repressor binding to its bipartite operator. CarS, produced on illumination, relieves repression of carB by physically interacting with CarA to dismantle CarA-DNA complexes. Here, we demonstrate that the N- and C-terminal portions of CarA are organized as distinct structural and functional domains. Specifically, we show that the 78 N-terminal residues of CarA, CarA(Nter), form a monomeric, highly helical, autonomously folding unit with significant structural stability. Significantly, CarA(Nter) houses both the operator and CarS binding specificity determinants of CarA. CarA(Nter) binds operator with a lower affinity than that for CarA(Nter)-CarS complex. Thus, the 78 N-terminal residues of CarA, CarA(Nter), form a monomeric, highly helical, autonomously folding unit with significant structural stability. Significantly, CarA(Nter) houses both the operator and CarS binding specificity determinants of CarA. CarA(Nter) binds operator with a lower affinity than whole CarA, and the CarA(Nter)-CarS complex has a 1:1 stoichiometry. In *vivo*, sufficiently high concentrations of CarA(Nter) block *M. xanthus* RNA polymerase-promoter binding, and this is relieved by CarS. In *vivo*, substitution of the gene *carB* by that for CarA(Nter) results in constitutive expression of carB just as in a *carA*-deleted background. However, re-engineering the latter strain to overexpress CarA(Nter) restores repression of *carB*. Thus, the 78-residue N-terminal portion of CarA is an autonomously folded, dual function domain that orchestrates specific DNA-protein and protein-protein interactions and, when overexpressed, can be functionally competent in *vivo*.

Light induces the synthesis of carotenoids in the Gram-negative bacterium *Myxococcus xanthus* (1). The carotenoids quench singlet oxygen and other free radicals produced upon illumination and thereby protect cells against photo-oxidative damage (2, 3). Two distinct genetic loci express the enzymes involved in carotenogenesis in *M. xanthus*; gene *crtI* encodes one enzyme, and the unlinked *carB* operon encodes the rest (4, 5). The photoinducible expression of these genes is controlled by several novel transcription factors (Fig. 1). In the dark, the extracytoplasmic function σ-factor CarQ is sequestered by the membrane protein CarR, whereas light triggers the inactivation of CarR to release CarQ (6, 7). The mechanism of the light-induced inactivation of CarR remains to be elucidated, but it involves at least one other protein factor, CarF (8).

Expression of *crtI* and the *carQRS* operon (to produce CarQ, CarR, and CarS-9) is then activated by the freed CarQ in conjunction with two constitutively expressed transcriptional factors: CarD, which resembles eukaryotic high mobility group A proteins (10–13) and the histone-like HfA (14). CarA, produced in a light-independent manner from an unlinked operon, and CarS, produced upon illumination, control the photoinduced expression of the *carB* operon (15–17). In the dark, the sequence-specific binding of CarA to its bipartite operator represses *carB* by blocking promoter access to RNA polymerase (17). CarS produced upon exposure to light causes derepression of *carB* by physically interacting with CarA to disrupt the CarA-DNA complexes (16, 17).

The present study is a molecular dissection of the structural and functional domains of CarA aimed at elaborating the molecular bases for the protein-protein and protein-DNA interactions involving the CarA-CarS pair. Our earlier yeast two-hybrid analysis had suggested that the 78 N-terminal residues of CarA, CarA(Nter), contain the regions mediating the interactions with CarS and that the remaining 209-residue C-terminal portion, CarA(Cter), was involved in CarA oligomerization (16). The ability of each of these two segments of CarA to mediate specific protein-protein interactions hinted that they could fold independently to the required conformation in the hybrid proteins employed. In the present study, we show that CarA(Nter) does indeed constitute an autonomously folding unit. Employing purified proteins, we confirm the existence of the physical interactions between CarA(Nter) and CarS, and establish the stoichiometry of the CarA(Nter)-CarS complex. We further demonstrate that CarA(Nter) is the sequence-specific DNA-binding domain of CarA, in accord with sequence analysis that hinted at a bacterial MerR-type helix-turn-helix DNA-binding motif in this domain (15, 18). We also provide evidence that CarA(Nter) can inhibit RNA polymerase-promoter binding *in vitro* and that this inhibition is removed by the association of CarA(Nter) with CarS. The dual ability of CarA(Nter) to exhibit specific protein-DNA as well as protein-protein interactions is a remarkable feature of this domain of the CarA repressor. Given that key properties of CarA reside in CarA(Nter), we examined the consequences in *vivo* of substituting CarA by CarA(Nter) and...
CarA N Terminus Mediates Operator and Antirepressor Binding

![schematic summary of known details of the light-induced carotenogenesis in *M. xanthus*. Genes are labeled and indicated by the short open arrows, proteins are shown as ovals, positive regulation is shown by continuous arrows, and negative regulation is shown by blunt-ended lines. The dotted arrows point to the corresponding gene product. Carotenoid biosynthesis enzymes are encoded by the gene *crtI* and the *carR* operon, whose respective promoters P<sub>I</sub> and P<sub>B</sub> are light-inducible. The *carA* operon contains the gene encoding the constitutively expressed CarA. *carQRS* expresses CarQ (an extracytoplasmic function σ factor), CarR (a membrane-bound anti-σ factor), and CarS. Other essential protein factors are the constitutively expressed CarD (a high mobility group A-like protein), IhdA (integration host factor α-subunit), and CarF (a putative membrane protein).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Wild-type *M. xanthus* strain DK1050 (19) and its derivative MRS44 carrying a nonpolar deletion within *carA* (15) were grown in the rich CTT medium (20). Plasmid constructions were performed in *Escherichia coli* strain DH5α and protein production in BL21-(DE3)pLysS, both grown in LB (21).

**Protein Overexpression and Purification**—Standard protocols were followed for DNA manipulation (21). Vector pET15b was used to overexpress proteins with His<sub>6</sub> tag (22). Using pMAR172 (see above) as template and appropriately designed primers, we PCR-amplified DNA fragments coding for (i) CarA(Nter), with the initiator ATG codon of CarA forming part of an NdeI site and with a stop codon at position 79 followed by an XhoI site, and (ii) CarA(Cter), corresponding to the region coding for the CarA C-terminal residues 80–288, immediately preceded by an NdeI site (that provides the initiator ATG codon) and with an XhoI site following the stop codon. The fragments were purified and cloned into the NdeI-XhoI sites of pET15b, and the constructs were verified by DNA sequencing.

The proteins used in the present study were all expressed as soluble proteins except for the partly soluble His<sub>6</sub>-CarA. Native His<sub>6</sub>-tagged CarA, CarS, and CarS1 were overexpressed and purified as previously described (16). His<sub>6</sub>-CarA(Nter) and His<sub>6</sub>-CarA(Cter) were purified using TALON metal affinity resin, and the native purification protocol at neutral pH, with imidazole elution (Clontech). His<sub>6</sub>-CarA(Nter) was further purified by reverse phase chromatography using a SourceRPC column in an AKTA HPLC apparatus (Amersham Biosciences) and lyophilized, and its identity was confirmed by matrix-assisted laser desorption ionization mass spectrometry. The yield per liter of cell culture was 10–20 mg of protein. CarA(Nter) and CarS were obtained by cleaving the His<sub>6</sub>-tagged proteins with thrombin at a 1000-fold dilution for 6 h in 150 mM NaCl, 50 mM phosphate buffer, pH 7.5, 5 mM β-mercaptoethanol. The reaction was quenched with phenylmethylsulfonyl fluoride and benzamidine, and the His tag peptide was removed by reverse-phase HPLC or extensive dialysis. Protein concentrations were determined from the absorbance at 280 nm in 6 M GdmCl using the following extinction coefficients, ε<sub>280</sub>: CarA(Nter) (1 Trp, 3 Tyr), 9540 M<sup>−1</sup> cm<sup>−1</sup>; CarA (4 Trp, 6 Tyr), 30,940 M<sup>−1</sup> cm<sup>−1</sup>; CarS (1 Trp and 1 Tyr), 6980 M<sup>−1</sup> cm<sup>−1</sup>; CarS1 (1 Tyr), 1490 M<sup>−1</sup> cm<sup>−1</sup> (23). Pure *M. xanthus* RNA polymerase holoenzyme was obtained as described elsewhere (17).

**Analytical Ultracentrifugation**—Sedimentation equilibrium experiments were carried out in a Beckman XL-A analytical ultracentrifuge and a Ti-60 rotor with six-sector Epon charcoal centerpieces of 12-mm optical path length. CarA(Nter) and His<sub>6</sub>-CarA(Nter) were dialyzed against 150 mM NaCl, 50 mM phosphate (pH 7.5), 1 mM β-mercaptoethanol using 3500-dalton cut-off dialysis tubing with three changes of buffer (each for >8 h). 70 μl of the pure dialyzed protein at 150–250 μM was centrifuged at 20 °C at 20,000 rpm (50,000 rpm for the base line) to equilibrium (verified when consecutive scans acquired in 2-h intervals were superimposable). Data were fit to the equation for an ideal solution with a single species (24) using EQASSOC (Beckman) to obtain apparent weight-average molecular weight. Partial specific volumes, υ<sub>i</sub>, calculated from the amino acid composition, were 0.728 ml/g for His<sub>6</sub>-CarA(Nter) and 0.732 ml/g for CarA(Nter) (25).

**Size Exclusion Chromatography**—Analytical gel filtration was carried out in an AKTA HPLC unit, using a Superdex-200 column (Amersham Biosciences). 100 μl of 5–50 μg pure protein or of a preincubated mixture were injected into the column equilibrated with 150 mM NaCl in buffer A, and the elution was tracked by absorbances at 280, 235, and 220 nm at flow rates of 0.3 ml/min. The column was calibrated using the following as standards (all from Sigma): cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and blue dextran (2 MDa) to determine the void volume (V<sub>v</sub>) and vitamin B<sub>12</sub> (1.35 kDa) to estimate total bed volume, V<sub>t</sub>. The calibration curve was as follows: log M<sub>r</sub> = 7.92–0.206 V<sub>t</sub> (correlation coefficient ≥ 0.99). V<sub>v</sub>, the elution volume, was assigned for each peak after verifying its identity in 15% SDS-polyacrylamide gels.

**Circular Dichroism and Fluorescence Spectroscopy**—CD and fluorescence measurements were done in an Applied Photophysics (UK) Picart apparatus coupled to a Peltier temperature control device and a Neulab RTE-70 water bath. CD calibration was with (+)-10-camphorsulfonic acid (26). Sample mixing and temperature measurement in the cuvette were achieved with magnetic stirrer and thermosensor accessories, respectively. Far-UV CD data were collected using 5–40 μg pure protein in 1- or 10-mm path length cuvettes and a slit width of 2 nm and averaged over four scans for CD spectra acquired with adaptive sampling or 10 repeats for the CD signal at 222 nm. A 10-mm path length cuvette, 5–10 μg protein, 280-nm excitation wavelength, and a slit
width of 5 nm were used for recording intrinsic fluorescence emission spectra with an emission slit width of 9.5 nm (averaged over three scans). Thermal denaturation data were recorded in 0.2 °C steps over a 90 °C temperature range using the far-UV CD signal at 222 nm, the sample overlaid with mineral oil to minimize evaporation. Data acquired with a zero settling time and a 2-h duration and with a 10-s settling time and a 5-h period yielded coincident traces. Curve fittings and the reported errors for a 5-min or overnight equilibration. Consequently, titrations were carried out by serial addition of small aliquots of a concentrated denaturant stock solution to the sample in the cuvette and recording the spectroscopic signal after a 5-min equilibration period with stirring. Each denaturation experiment was repeated twice.

A two-state model for the N (native) = U (unfolded) equilibrium with an apparent equilibrium constant \( K_C = [U]/[N]\) was used in analyzing denaturation data. \( K_C = f_{N}f_{U} / f_{N}f_{U}\), where \( f_{N} \) and \( f_{U} \) are, respectively, the fractions of unfolded and native protein at a given point in the denaturation curve. The free energy of unfolding, \( \Delta G_U \), is assumed to be linear with denaturant concentration (Equation 1), and its temperature dependence is expressed in terms of a modified form of the Gibbs-Helmholtz equation (Equation 2; see Ref. 28).

\[
\Delta G_U = -RT\ln \left( \frac{f_{N}}{f_{U}} \right) = -RT\ln \left( \frac{[N]}{[U]} \right) = -RT\ln \left( \frac{K_C}{1 + K_C} \right)
\]

or

\[
\Delta G_U = -RT\ln \left( \frac{f_{N}}{f_{U}} \right) = -RT\ln \left( \frac{[N]}{[U]} \right) = -RT\ln \left( \frac{K_C}{1 + K_C} \right)
\]

\[
\Delta G_U(T) = \Delta G_U(37°C) + \Delta C_P(T - 37°C)
\]

\( \Delta C_P \) is the pre- and post-transition “base lines,” are described as linear functions of temperature or denaturant concentration. Urea or GdmCl denaturation curves were fit to Equation 1 with \( m, C_m \), and \( m \), and four parameters for the pre- and post-transition base lines as variables. Thermal denaturation curves were fit to Equation 2 by fixing \( \Delta C_P \) and varying \( \Delta H_m \) and \( T_m \), the heat capacity, and \( m \), the denaturation dependence, are assumed to be temperature-independent. The observed \( (y) \), native \( (y_N) \), and unfolded \( (y_U) \) CD or fluorescence signal at a given temperature (\( T \), in Kelvin) or denaturant concentration yield \( \Delta G_U \), as follows.

\[
\Delta G_U = -RT(y_N - y_U)
\]

\( y_N \) and \( y_U \) are the pre- and post-transition “base lines,” are described as linear functions of temperature or denaturant concentration. Urea or GdmCl denaturation curves were fit to Equation 1 with \( m, C_m \), and \( m \), and four parameters for the pre- and post-transition base lines as variables. Thermal denaturation curves were fit to Equation 2 by fixing \( \Delta C_P \) and varying \( \Delta H_m \) and \( T_m \), the heat capacity, and \( m \), the denaturation dependence, are assumed to be temperature-independent. The observed \( (y) \), native \( (y_N) \), and unfolded \( (y_U) \) CD or fluorescence signal at a given temperature (\( T \), in Kelvin) or denaturant concentration yield \( \Delta G_U \), as follows.

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\[
\Delta G_U = -RT(y_N - y_U)
\]
RESULTS

CarA(Nter) Is a Stably Expressed, Compact, Monomeric Domain of CarA—CarA(Nter), the 78 N-terminal residues of CarA, shows sequence similarity to the winged helix domain in bacterial MerR proteins (15, 18) (Fig. 2), where it appears as an independent module in the structures available for members of this family (35–37). Sequence analysis also revealed the similarity of the remaining 209 C-terminal residues of CarA, CarA(Cter), to the domain for cobalamin-binding (15) such as in methionine synthase, where it occurs as an autonomous unit (38). The charged residue distribution in CarA is consistent with its structural and functional compartmentalization into CarA(Nter) and CarA(Cter); CarA(Nter) is highly basic, with a calculated pl of 10.68, whereas CarA(Cter) is acidic, with a calculated pl of 5.78. As mentioned earlier, each of these two CarA fragments exhibited specific protein-protein interactions when analyzed in the yeast two-hybrid system, CarA(Nter) with CarS and CarA(Cter) with CarA, implying that each folds correctly in the hybrid proteins (16).

We could overexpress both CarA(Nter) and CarA(Cter) in E. coli as soluble proteins and purify them in milligram quantities consistent with these being stable, independently folding domains (39). The oligomeric states and compactness of these purified fragments were assessed by analytical ultracentrifugation and gel filtration. The calculated molecular weights for CarA(Nter) and His-tagged CarA(Nter) are 9.5 and 11.3 kDa, respectively. Sedimentation equilibrium data (Fig. 3) yielded a molecular mass of 10.3 ± 0.6 kDa for CarA(Nter) and 11.5 ± 1 kDa for His6-CarA(Nter), indicating these to be monomers. In analytical gel filtration HPLC using a Superdex-200 column, a single eluted peak (with sharpness comparable with any of the compact globular proteins used as standards) for CarA(Nter) indicates a homogeneously populated species, and molecular mass, estimated from the Vc, was 9.6 ± 0.3 kDa, as expected for a monomer (see below) (Fig. 5, bottom). An eluted peak was not detected for His6-CarA(Nter), indicating material loss possibly from precipitation or nonspecific binding to the matrix. His6-CarA(Cter) eluted off of the Superdex-200 column as a single peak with a slight shoulder on the lagging side (data not shown). Relative to the calculated molecular mass of 24.9 kDa, the mass for CarA(Cter) was 38.6 ± 1.2 Da as estimated from Vc, corresponding to the peak maximum. This suggests a monomer-dimer population distribution, the dimeric form being consistent with CarA-CarA(Cter) interactions observed in yeast two-hybrid analysis (16). Thus, CarA(Cter) appears to be a dimerization domain, whereas CarA(Nter) is a compact monomer. The remainder of this report focuses on further characterization of CarA(Nter); that for CarA(Cter) will be described elsewhere.

Folding and Thermodynamic Stability of CarA(Nter)—

Far-UV CD spectra for native CarA(Nter) and His6-CarA(Nter) have minima at 222 and 208 nm characteristic of predominantly α-helical proteins (Fig. 4A) (40). The mean residue ellipticity at 222 nm ([θ]222 in degrees cm2 dmol−1), which arises mainly from helical conformations, was −19,000 for CarA(Nter) at 25 °C but lower (−13,000) for His6-CarA(Nter), presumably due to contributions from the randomly structured His6 tag. For a polypeptide of chain length Nc, [θ]222 at 25 °C is 895 for 0% helix and can be expressed as (−37,750(1 − 3Nc)}.
for 100% helix (41). These yielded helix content estimates of 50% for CarA(Nter) and 33% for His<sub>6</sub>-CarA(Nter). The helix content thus estimated for native CarA(Nter) is close to that expected from the sequence alignment in Fig. 2, and the available three-dimensional structures of BmrR, MtaN, CueR, and ZnR (about half of the CarA(Nter) residues would adopt helical conformation). |θ|<sub>222</sub> for urea or GdmCl-denatured CarA(Nter) was close to zero as expected (Fig. 4A). The single Trp (Trp<sub>22</sub>) in native CarA(Nter) exhibited an intrinsic fluorescence emission maximum at 328 nm that was red-shifted to 346 nm and with a loss in intensity upon denaturing the protein (Fig. 4B). This is consistent with CarA(Nter) having a defined, compact tertiary structure in which its single Trp is buried in a solvent-excluded environment (40).

The structural stability of CarA(Nter), in terms of its free energy of unfolding ΔG<sub>U</sub>, was estimated by monitoring folding transitions using CD and intrinsic Trp fluorescence. Both thermally induced and urea (or GdmCl)-induced transitions were reversible and equilibrated rapidly. Analysis of urea denaturation curves (Equation 1) yielded essentially identical parameters (within experimental error) for the fluorescence and far-UV CD data, consistent with a two-state folding behavior and an average thermodynamic stability, ΔG<sub>U</sub>, of 4.42 ± 0.25 kcal mol<sup>−1</sup> (Fig. 4C, Table I). Analysis of the thermally induced folding transition (Fig. 4D) requires a knowledge of the heat capacity, ΔC<sub>p</sub>, that is accurately determined only by calorimetry or extensive denaturation data, both outside the scope of the present study (see Ref. 28). Based on average ΔC<sub>p</sub> per residue estimates from several other proteins (13.6 ± 2.2 to 14.2 ± 2.5 cal mol<sup>−1</sup> K<sup>−1</sup>) (28, 42, 43), ΔC<sub>p</sub> for CarA(Nter) is likely to be in the range of 0.9–1.4 kcal mol<sup>−1</sup> K<sup>−1</sup>. The thermal transition curve for CarA(Nter) can then be analyzed using Equation 2 and fixing ΔC<sub>p</sub> at 0.9 or 1.4 kcal mol<sup>−1</sup> K<sup>−1</sup> (assumed to be temperature-independent). Table I lists the values thus obtained for T<sub>m</sub>, ΔH<sub>m</sub>, and ΔG<sub>U</sub>(25 °C). These indicate that the true ΔC<sub>p</sub> for CarA(Nter) is probably close to 0.9 kcal mol<sup>−1</sup> K<sup>−1</sup>, since the corresponding estimate of ΔG<sub>U</sub>(25 °C) is close to that determined independently from urea denaturation data at this temperature. Overall, the two-state folding behavior and the thermodynamic stability of CarA(Nter) resemble those reported for a number of other small globular helical proteins or protein domains (see Refs. 28 and 44). Thus, CarA(Nter) is a stable, autonomously folding unit.

### Table I

| Method          | Urea denaturation<sup>a</sup> | Fluorescence<sup>b</sup> | Average<sup>c</sup> |
|-----------------|-------------------------------|-------------------------|---------------------|
|                 | m kcal mol<sup>−1</sup> M<sup>−1</sup> | u kcal mol<sup>−1</sup> | ΔG<sub>U</sub> kcal mol<sup>−1</sup> |
| Far-UV CD       | 0.78 ± 0.05                   | 5.59 ± 0.08             | 4.35 ± 0.25         |
| Fluorescence    | 0.85 ± 0.05                   | 5.32 ± 0.07             | 4.52 ± 0.24         |
| Average         | 0.82 ± 0.05                   | 5.45 ± 0.19             | 4.42 ± 0.25         |

<sup>a</sup> Solution conditions: 150 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5, 25 °C.

<sup>b</sup> Fixed at the value indicated for data fitting to Equations 3 and 4.

<sup>c</sup> Calculated using Equations 3 and 4 with fixed ΔC<sub>p</sub> values indicated and varying T<sub>m</sub> and ΔH<sub>m</sub> as the adjustable parameters.

The hybrid proteins of the yeast two-hybrid system, the CarA segment corresponding to CarA(Nter) interacted with CarS, as mentioned earlier (16). To test whether CarA(Nter) on its own can form stable complexes with CarS, we analyzed mixtures of the two purified proteins by HPLC in a Superdex-200 analytical gel filtration column (Fig. 5A). This can provide knowledge about the formation of any stable complexes as well as their stoichiometry. Purified CarA(Nter) eluted off an analytical Superdex-200 column as a compact monomer with a molecular mass of 9.6 kDa. Compared with the calculated value of 12.5 kDa, CarS eluted with a mass of 15.5 kDa, suggesting that it exists predominantly as a monomer (Fig. 5A, bottom). For mixtures of CarS and CarA(Nter), an additional peak eluted with an apparent molecular mass of 29.9 ± 2 kDa (Fig. 5A, top). This peak contained both CarA(Nter) and CarS in nearly equivalent amounts as verified by SDS-PAGE, and its mass would correspond to a 1:1 complex of the two proteins. Thus, CarA(Nter) on its own binds specifically to CarS to form a stable binary complex with a 1:1 stoichiometry. CarS<sub>1</sub> is a gain-of-function mutant of carS (33), which bears a mutation that replaces the codon for Trp in CarS with a stop codon (9). The resulting 86-residue CarS<sub>1</sub> lacks the last 25 C-terminal residues of CarS and when probed by yeast two-hybrid analysis was also found to interact with CarA (16). This indicated that the CarA-binding residues in CarS...
map to its first 86 residues. Here, we confirmed that CarA(Nter) can interact with purified His$_{6}$-CarS1 by analytical gel filtration using the Superdex-200 column (Fig. 5B). Pure His$_{6}$-CarS1 appears to be primarily monomeric, since it eluted with a mass of $15.1 \pm 0.1$ kDa compared with the calculated value of 11.5 kDa (Fig. 5B, bottom). The molecular mass of His$_{6}$-CarS1 relative to the calculated value is higher than for CarS, possibly because it is less compact, due to the flexible His$_{6}$-tag. When mixed with CarA(Nter), a new peak eluted with apparent mass of 27.2 $\pm$ 1.2 kDa (Fig. 5B, top) and contained CarA(Nter) and His$_{6}$-CarS1 in roughly equal amounts in an SDS-polyacrylamide gel. This indicates a 1:1 complex of the two proteins. There are observable differences in the individual peaks obtained for the CarA(Nter)-His$_{6}$-CarS1 complexes relative to those for CarA(Nter)-CarS. These may be due to the considerably lower extinction coefficient of His$_{6}$-CarS1 (1 Tyr) relative to those for CarA(Nter) (as in A) and His$_{6}$-CarS1. The bottom panels show the elution profiles for the pure proteins; the top panels show mixtures of the two proteins in equimolar amounts (a), or with a 2-fold (b) or 3-fold (c) molar excess of CarS or CarS1 over CarA(Nter). Note that the extinction coefficient at 280 nm is considerably lower for CarS1 (1 Tyr) than for CarS (1 Tyr and 1 Trp) as listed under Experimental Procedures. The apparent molecular weights calculated from the elution volumes are described in the text.

Further downstream (to position $-19$) when the CarA levels are raised.

Whether CarA(Nter) is also capable of binding to CCR and, if so, the nature of this binding were examined by gel retardation and DNase I footprinting. As shown in Fig. 6C, CarA(Nter) did bind to CCR but with lower affinity relative to CarA; comparable binding was observed at concentrations of CarA(Nter) over an order of magnitude greater than those of CarA. The retarded band at 370 nM CarA(Nter) (6-fold the maximum CarA concentration employed) exhibited a smearing out toward higher mobility complexes, possibly reflecting partial binding of CarA(Nter). The somewhat diffuse retarded band spreading toward slower mobility complexes at 1850 nM CarA(Nter) (30-fold the maximum CarA concentration used) may reflect additional nonspecific binding or some cooperativity in DNA-binding that occurs at these concentrations. However, in no case was a second, distinct retarded band observed with CarA(Nter), in contrast to CarA. The DNase I footprint (Fig. 6D) for the highest CarA(Nter) concentration used compared well with that observed for CarA in its span (from position $-70$ to $-26$) and in the hypersensitive sites observed ($-63$ and $-55$). However, with CarA(Nter) the DNase I hypersensitivity at $-55$ was far more pronounced, and additional hypersensitive sites appeared at $-56$ and $-35$ that were not seen with CarA. DNase I acts by binding to the minor groove, and sites hypersensitive to its action are generally interpreted as sites of local bending of the protein-bound DNA toward the major groove leading to a widened and more DNase I-susceptible minor groove (45). The similar pattern of hypersensitive sites obtained for CarA(Nter) and CarA suggests that both bring about nearly equivalent conformational changes in the specific DNA stretch to which they bind. The additional hypersensitive sites with CarA(Nter) may reflect a greater accessibility to DNase I stemming from its smaller size relative to CarA. Nevertheless, the overall features of the footprint for CarA(Nter) resemble those for CarA. The observation that sufficiently high concentrations of CarA(Nter) nearly mimic the intact protein in the specific binding to DNA demonstrates that the DNA binding specificity determinants of CarA are housed in CarA(Nter).
CarS Destabilizes CarA(Nter)-DNA Complexes to Facilitate RNA Polymerase-Promoter Binding in Vitro—We established above that at sufficiently high concentrations of CarA(Nter), its binding to the CarA operator in vitro resembles that observed with the whole protein. Moreover, CarA(Nter) on its own binds to CarS. As shown in an electrophoretic mobility shift assay of His<sub>6</sub>-CarA binding to the 130-bp CCR probe, with the increasing protein concentrations shown at the top, C, same as B but with His<sub>6</sub>-CarA(Nter). D, DNase I footprinting of His<sub>6</sub>-CarA(Nter) bound to probe CCR (lanes 1–5). Protection against DNase I is shown by a solid line, and hypersensitive sites are shown by arrowheads on the right. The DNase I footprint obtained with the highest concentration of His<sub>6</sub>-CarA used is shown in lane 6 for comparison. The locations corresponding to the CarA operator sites pl and pII and the −35 promoter region are marked on the left. G + A and C + T chemical sequencing ladders of the 130-bp fragment (not shown) were used in assigning the footprints. Other solution conditions are described under “Experimental Procedures.”

Fig. 6. Binding of CarA(Nter) to probe CCR containing the carB promoter-operator region. A, promoter-operator region of the carB operon. DNA sequence features of the segment from position +1 (the transcriptional start point) to position −70 are shown. The −35 and −10 promoter elements corresponding to consensus sequences are underlined. pl and pII are the high and low affinity operator sites, respectively. B, electrophoretic mobility shift assay of His<sub>6</sub>-CarA binding to the 130-bp CCR probe, with the increasing protein concentrations shown at the top. C, same as B but with His<sub>6</sub>-CarA(Nter). D, DNase I footprinting of His<sub>6</sub>-CarA(Nter) bound to probe CCR (lanes 1–5). Protection against DNase I is shown by a solid line, and hypersensitive sites are shown by arrowheads on the right. The DNase I footprint obtained with the highest concentration of His<sub>6</sub>-CarA used is shown in lane 6 for comparison. The locations corresponding to the CarA operator sites pl and pII and the −35 promoter region are marked on the left. G + A and C + T chemical sequencing ladders of the 130-bp fragment (not shown) were used in assigning the footprints. Other solution conditions are described under “Experimental Procedures.”
(lane 2), and this disappeared in excess CarS (lane 3). For these conditions, CCR-\textit{M. xanthus} RNAP binding was observed only in the absence of CarA (compare lanes 4 and 5) or with excess CarS also present (lane 6). The assay carried out with CarA(Nter) at sufficiently high concentrations gave parallel results. Stable CarA(Nter)-CCR binding (lane 8) was abolished by CarS (lane 9). Moreover, \textit{M. xanthus} RNAP-CCR complex formation was deterred by CarA(Nter) (compare lane 11 with lane 4 or 10), as with CarA. Again, the additional presence of CarS restored stable \textit{M. xanthus} RNAP-CCR binding (lane 12). In short, CarA(Nter), at sufficiently higher concentrations, emulates intact CarA in preventing \textit{M. xanthus} RNAP binding at the \textit{carB} promoter (repression), and this is counteracted when CarS is also present (antirepression).

**Overexpression of CarA(Nter) in Vivo Represses carB**—Both the DNA-binding and CarS-binding functions of CarA reside in CarA(Nter), and, under suitable conditions, the interplay between \textit{M. xanthus} RNAP, CarA, and CarS at P_\text{B} can apparently be reproduced by CarA(Nter), as described above. So, we examined whether CarA can be substituted for by CarA(Nter) in \textit{M. xanthus}. Wild-type DK1050 \textit{M. xanthus} cell colonies are yellow in the dark but become red when illuminated with blue light due to the production of carotenoids. By contrast, colonies of a \textit{M. xanthus} strain with carA deleted (MR844) are orange in the dark because \textit{carB} is no longer repressed and become red in the light due to the photoinduced expression of \textit{crtI}. This color phenotype provides a direct, visual means by which functional CarA can be distinguished from lack-of-function variants \textit{in vivo}.

We first examined whether CarA(Nter) can substitute for CarA when its expression is governed in the same genetic context as the whole protein. For this, we engineered an \textit{M. xanthus} mutant in which all but the coding region for CarA(Nter) was deleted from the \textit{carA} gene as described under “Experimental Procedures.” This mutant exhibits the color phenotype of the \textit{carA}-deleted mutant strain MR844 (i.e. orange in the dark with the color intensifying in light) (Fig. 8A). This suggests that, when expressed in its natural context, the C-terminal region is absolutely required for CarA to be functionally competent \textit{in vivo}. This may be a reflection of CarA(Nter) levels being insufficient for effective repression of \textit{carB} under these conditions.

We therefore studied the consequences of overexpressing CarA(Nter) in a carA-deleted background. To achieve this in \textit{M. xanthus}, we engineered gene \textit{carA(Nter)} to be under the control of the 16 S ribosomal RNA promoter, since such bacterial promoters are exceptionally strong. They usually occur as two tandemly repeating promoters, each having an exact or nearly exact match to the consensus 5’-H11002 10 hexamer sequences (46). We generated constructs in which CarA(Nter) expression was placed under the control of (i) the entire 16 S rRNA promoter, 2P_rRNA::carA(Nter) or (ii) the downstream of
A

| Strain   | Description  | Carotenoid Phenotype | Dark | Light |
|----------|--------------|---------------------|------|-------|
| DK1050  | Wild type    | Yellow              | Red  |       |
| MR864   | ΔcarA        | Orange              | Red  |       |
| MR1719  | carA(Nter)   | Orange              | Red  |       |
| MR1720  | 1PrRNA::carA(Nter) | Light Orange | Red  |       |
| MR1721  | 2PrRNA::carA(Nter) | Yellow          | Yellow |     |

B

**Thick line** bold

**Thin line**

**Color phenotype of M. xanthus strains expressing CarA(Nter)**

The two tandem promoters of the 16 S rRNA promoter, 1PrRNA::carA(Nter). The constructs were separately incorporated at a heterologous site in the carA-deleted strain, as described under “Experimental Procedures.” The constitutive phenotype for carotenogenesis was barely altered when 1PrRNA::carA(Nter) was used for expressing CarA(Nter) in vivo, possibly because CarA(Nter) is still not produced in amounts that exceed those of CarS so as to effectively shut off carB expression in vivo. The observed observations were further reinforced by measuring β-galactosidase levels expressed off of a reporter lacZ gene under carB promoter control (Fig. 8B). Complete deletion of carA (strain MR1744) showed the expected high levels of β-galactosidase expression in the dark relative to the control strain MR418. By contrast, when CarA(Nter) was produced from the complete 16 S rRNA promoter (MR1747), β-galactosidase levels in the dark were even lower than for the control. These results indicate a dosage-dependent repression of P_b by CarA(Nter) in vitro, consistent with the observation in vitro that sufficiently high levels of CarA(Nter) prevent M. xanthus RNAP binding to P_b. As expected, in view of the color behavior summarized in Fig. 8A, low β-galactosidase levels for MR1747 persisted even upon exposure to light, suggesting that CarA(Nter) is superproduced to such an extent that its repressor activity cannot be circumvented by the photoinduced CarS. From the above results, it may also be concluded that the role of the C-terminal portion of CarA is presumably in enhancing operator binding affinity for effective repression of carB under normal (wild-type) conditions in vivo.

**DISCUSSION**

The specific binding of CarA to its operator represses the carB operon, and this occurs in the absence of light. CarS produced on illumination binds specifically to CarA, thereby leading to the derepression of carB. The present study provides insights into the domain organization of CarA and has permitted a mapping of the specific binding activities of CarA to its operator DNA and to the CarS protein. We found that both these activities localize to the first 78 N-terminal residues of the protein, CarA(Nter), whereas the remaining C-terminal part is involved in oligomerization.

The data presented here show that CarA(Nter) is a stable, autonomously folding unit of the protein. CarA(Nter) is monomeric with a high α-helical content and has a hydrodynamic behavior expected for a compact globular protein. As an autonomously folding domain, CarA(Nter) exhibits cooperative and reversible thermally or chemically induced folding transitions. The coincident equilibrium folding transitions for CarA(Nter) with different spectroscopic probes (far-UV CD for secondary structure and intrinsic Trp fluorescence for tertiary structure) indicate that these transitions can be quantitatively described by a two-state model. This is the case for several small, stable proteins and protein domains (28, 44). Like these, CarA(Nter) has high thermal stability (T_m) and modest thermodynamic stability (ΔG^o_f). That this autonomously folding region is also functionally competent is shown by CarA(Nter) exhibiting the sequence-specific DNA-binding characteristic of the whole protein (albeit with a lower affinity) and being able to repress carB in vivo under suitable conditions. This is also in accord with the highly basic nature of this segment of the protein and the predicted sequence similarity to the DNA-binding region of the bacterial MerR protein family. The available high resolution, three-dimensional structures of MerR-type proteins show this domain to have a high helix content and to belong to the winged helix family of protein structures (35–37). The helical content for CarA(Nter) estimated from CD data agrees well with that expected if its structure resembled the ones available for proteins of the MerR family. The observation that repressor activity in vivo by CarA(Nter) requires it to be abundant implies that this domain, although sufficient for function, acts less efficiently than the whole protein. This is consistent with intact CarA having a higher operator-binding affinity that is attributable to its acidic C-terminal portion, CarA(Cter), which we find to be stably folded, largely dimeric, and thus involved in oligomerization.

The observed domain organization for CarA resembles that of various prokaryotic repressors that use the helix-turn-helix motif to bind DNA and do so as homodimers, with each motif...
binding to one half-site of symmetry-related DNA (47). CarA is also likely to use this motif for DNA binding, and it does have a high affinity operator site with dyad symmetry. The modular division of the DNA binding and oligomerization activities in these repressor proteins provides a remarkably efficient control of gene expression by thermodynamically linking protein-protein and protein-DNA binding. The bipartite nature of the CarA operator, with a high affinity and a low affinity site, and the observed cooperative binding of CarA to its operator emphasizes the importance of the cross-talk between DNA-bound CarA molecules. Thus, as we have shown, CarA(Nter) is no substitute for CarA in repressing carB in vivo under normal levels of expression. Nevertheless, overexpression of CarA(Nter) can repress carB in vivo, and sufficiently high concentrations of CarA(Nter) can impede RNA polymerase binding to the carB operator-promoter region in vitro. Whether the C-terminal segment of CarA plays roles other than in fostering binding cooperativity remains an open question. The oligomerization domain in a number of other bacterial repressors, including the MerR family, frequently binds cofactors and thus provides an additional level of combinatorial control. Sequence analysis has revealed that the CarA C-terminal segment contains the signature sequence for vitamin B12 binding (15). We have observed that CarA(Cter) does bind to this cofactor in vitro (data not shown); but, as shown in this study, this domain can be dispensed with under suitable conditions. A detailed analysis of the role of this cofactor binding in CarA function is currently being pursued and will be presented elsewhere.

The switch that turns off CarA-directed repression of carB is the binding of CarS to CarA, as described earlier. We have shown in this study that the antirepressor CarS interacts physically with the same subdomain of CarA as that involved in its specific binding to DNA. CarA(Nter)-CarS complexes have a 1:1 stoichiometry, and their formation eliminates DNA binding by CarA(Nter) and, as a consequence, restores RNA polymerase-promoter binding. We find that CarS in amounts equal to CarA(Nter) is sufficient for disrupting operator-CarA(Nter) complexes in vitro, in contrast to the severalfold excess of CarS over CarA required to disrupt operator-CarA complexes. Thus, CarS is far more effective against operator-binding by CarA(Nter) than by CarA. This can be attributed to the greater affinity of CarA for operator DNA. Consequently, the mechanism for the P1 derepression in the light appears to be one of direct competition between CarS and DNA for binding to the same subdomain of CarA, as summarized in Fig. 9. Derepression could arise from a higher affinity of CarS for CarA relative to that of the operator and/or from the high levels of CarS produced upon photoinduction. The CarS and DNA binding sites on CarA could be distinct but located sufficiently close to one another, or these sites could overlap completely or partially, such that binding is mutually exclusive. If the latter is the case, it is tempting to speculate that CarS might structurally mimic the operator DNA in size, shape, and electrostatic complementarity. This could result if the negatively charged carboxylate groups of aspartate and glutamate residues in CarS are juxtaposed in a manner that resembles the phosphate of the DNA backbone. Examples of DNA-associated systems where the DNA is structurally mimicked by α-helices and/or β-sheets include phage T7 Ocr, which “antirestricts” type I restriction enzymes (48); the N terminus of eukaryotic TATA box-binding protein-associated factor TAF1230 (49); and phase PBS1 uracil-DNA glycosylase inhibitor (50). Like these DNA mimics, CarS and CarS1 are also highly acidic. We are currently investigating whether any of the DNA-binding residues in CarA are also involved in CarS binding as well as the attractive possibility that CarS is a DNA structural mimic.

**Fig. 9. Model for CarA-mediated repression and antirepression by CarS.** The N-terminal domain of CarA, the C-terminal domain of CarA, CarS, and M. xanthus RNA polymerase, are indicated by N, C, S, and MxRNAP, respectively. CarA oligomers, possibly dimers, form through CarA(Cter) interactions. Stepwise binding (represented by the curved unfilled arrow) of dimeric CarA to its bipartite operator (thick arrows) through CarA(Nter) represses carB in the dark. CarS produced by light interacts with CarA(Nter), to abolish operator binding and so to derepress carB. The schematic depiction of overlapping or identical CarA(Nter) binding surfaces for DNA or CarS remains to be experimentally confirmed.

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The N Terminus of *Myxococcus xanthus* CarA Repressor Is an Autonomously Folding Domain That Mediates Physical and Functional Interactions with Both Operator DNA and Antirepressor Protein

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