J. Mol. Biol. (1998) **278**, 915–933

JMB

Association States of the Transcription Activator Protein NtrC from *E. coli* Determined by Analytical Ultracentrifugation

Karsten Rippe*, Norbert Mücke and Alexandra Schulz

Deutsches Krebsforschungszentrum Abteilung Biophysik der Makromoleküle, Im Neuenheimer Feld 280 D-69120, Heidelberg, Germany

*Corresponding author

The transcription activator protein NtrC (nitrogen regulatory protein C) can catalyze the transition of *E. coli* RNA polymerase complexed with the σ^{54} factor (RNAP·σ^{54}) from the closed complex (RNAP·σ^{54} bound at the promoter) to the open complex (melting of the promoter DNA). This process involves phosphorylation of NtrC, assembly of a multimeric NtrC complex at the enhancer DNA sequence, interaction of this complex with promoter bound RNAP·σ^{54} *via* DNA looping, and hydrolysis of ATP. We have used analytical ultracentrifugation to study the different NtrC association states and to derive hydrodynamic models for the conformation of the various NtrC species. The following results were obtained. (i) The unphosphorylated wild-type protein formed a dimer with a measured molecular weight of 102(±3) kDa, which compares to a calculated molecular weight of 54 kDa for a monomer (concentration range studied 2 to 8 μM NtrC monomer). (ii) In the unphosphorylated state one NtrC dimer was bound to one binding site as determined with DNA oligonucleotide duplexes containing one or two binding sites (concentration range studied 50 to 1000 nM NtrC dimer). (iii) The data obtained at protein concentrations that were below the concentration of binding sites indicate that binding to the DNA duplex with two binding sites occurred with essentially no cooperativity. The experiments were conducted in the absence of ATP. (iv) The phosphorylated protein formed a specific complex at the DNA duplex with the enhancer sequence (two NtrC binding sites) that consisted of four dimers (concentration range studied 100 to 1000 nM NtrC dimer). (v) The formation of this octameric complex was highly cooperative, and the data suggest that two DNA strands could bind simultaneously to this complex. (vi) From the sedimentation data a model was derived in which the NtrC dimer adopts a V shaped structure with the DNA binding domains being located at the bottom and the two receiver domains at the top of the V. In this conformation higher order NtrC complexes can be stabilized by interaction between the phosphorylated receiver domain and the central activation domain of different NtrC dimers.

Keywords: enhancer; protein-DNA interaction; transcription; RNA polymerase

Introduction

Nitrogen regulatory protein C (NtrC, also designated nitrogen regulator I or NR_I) from *Escherichia coli* is one of the activator proteins of *E. coli* RNA polymerase σ^{54} holoenzyme (RNAP·σ^{54}) for a variety of genes that are involved in nitrogen utilization (Magasanik, 1996; North *et al.*, 1993). The distal location of the NtrC binding sites found

Abbreviations used: NtrC, nitrogen regulatory protein C; NtrC-P, phosphorylated NtrC; RNAP·σ^{54}, *E. coli* RNA polymerase σ^{54} holoenzyme; NtrB, nitrogen regulatory protein B; AUC, analytical ultracentrifugation; *M*, molecular weight; *s*, sedimentation coefficient; *D*, diffusion constant; BSA, bovine serum albumin.

0022–2836/98/200915–19 $25.00/0/mb981746

in vivo requires looping of the intervening DNA to interact with RNAP·σ^{54} at the promoter (Gralla & Collado-Vides, 1996; Reitzer & Magasanik, 1986; Rippe *et al*., 1997; Su *et al*., 1990; Wedel *et al*., 1990). As deduced from gel filtration experiments, unphosphorylated NtrC forms a dimer free in solution, and it has been concluded from gel electrophoretic analysis that a dimer is bound to a single NtrC binding site on the DNA (Klose *et al*., 1994; Porter *et al*., 1993; Reitzer & Magasanik, 1983).

Wild-type NtrC is phosphorylated *in vivo* by the NtrB protein (nitrogen regulatory protein B, also designated nitrogen regulator II or NR_{II}) at Asp54 which greatly increases the cooperativity of DNA binding to two sites (Chen & Reitzer, 1995; Porter *et al*., 1993; Weiss *et al*., 1992) and activates the ATPase function of NtrC (Weiss *et al*., 1991). The same phosphorylation of NtrC can be reproduced *in vitro* by the addition of a phosphorylating chemical like carbamyl phosphate ($CH_2NO_5PNa_2$) in the presence of $MgCl_2$ (Feng *et al*., 1992). The phosphorylation state of NtrC and its ability to form larger complexes at the enhancer appear to be interconnected (Porter *et al*., 1993; Weiss *et al*., 1992; Wyman *et al*., 1997). Furthermore, it has been shown in a number of studies that the unphosphorylated protein cannot activate transcription, and deletion of the second NtrC binding site leads to a large reduction in the activation rate (Austin & Dixon, 1992; Mettke *et al*., 1995; Porter *et al*., 1993; Weiss *et al*., 1991, 1992). Thus, oligomerization of phosphorylated NtrC dimers at the enhancer is required for the formation of an active NtrC complex and appears to be a crucial feature of its biological function.

Higher order complexes of NtrC dimers are too unstable to be studied by gel electrophoresis (Porter *et al*., 1993), but they have been observed by electron microscopy (Porter *et al*., 1993; Révet *et al*., 1995; Su *et al*., 1990) and scanning force microscopy (Rippe *et al*., 1997; Wyman *et al*., 1997). For the constitutively active mutant $NtrC^{S160F}$ it has been demonstrated by analytical ultracentrifugation that the free protein oligomerizes to form a higher order state like a hexamer or an octamer in the presence of ATPγS (Farez-Vidal *et al*., 1996). In addition, the results of fluorescence anisotropy measurements indicate that this holds true also for the complex of $NtrC^{S160F}$ with the enhancer DNA (Sevenich *et al*., 1998). A quantitative analysis by scanning force microscopy in air revealed that 30% of the complexes formed with phosphorylated NtrC (NtrC-P) at the enhancer were larger than two dimers, and it was shown that oligomerization of NtrC-P is required for transcriptional activation (Wyman *et al*., 1997). These results have led to the idea that NtrC binds as an octameric complex at the enhancer upon phosphorylation of the protein (Wyman *et al*., 1997).

However, with the above mentioned methods it has not been possible to determine the exact size of the active NtrC complex formed upon phosphorylation at the enhancer DNA sequence under native equilibrium conditions free in solution. As this is a central point for understanding the mechanism by which NtrC functions as a transcription activator, we have used analytical ultracentrifugation (AUC) to study the different association states of NtrC. AUC is a well established method to measure the absolute molecular weight M of a protein or a protein-DNA complex very accurately in sedimentation equilibrium runs. In addition, hydrodynamic parameters like the sedimentation coefficient s and the diffusion constant D can be determined by sedimentation velocity analysis. For recent reviews on the method see Hensley (1996), Laue (1995), Schuster & Toedt (1996), Stafford (1997).

Results

Characterization of NtrC protein preparation

To provide a simple and efficient protein purification procedure for the NtrC protein the gene for NtrC was cloned in pET15b from Novagen and over-expressed with a N-terminal His-tag (see Materials and Methods). This protein was used in all the experiments described below. After purification no contaminating bands were detectable on a Coomassie stained SDS-gel (data not shown) and the purity was estimated to be >95%. The protein preparation was tested in gel-shift assays for specific binding to the DNA oligonucleotide duplexes ES-1 and ES-2 (data not shown), the sequence of which is given in Figure 1. The NtrC binding site in these duplexes corresponded to the high affinity NtrC binding site Lp overlapping the promoter for *ntrB* (*glnL*) (Magasanik, 1996). The 32 bp DNA duplex ES-1 contained one binding site, and the 59 bp DNA duplex ES-2 had two of these binding sites (Figure 1).

The activity of the His-tagged NtrC protein preparation studied here was confirmed with the plasmid pJES534 in an *in vitro* transcription assay (Klose *et al*., 1993; Porter *et al*., 1993). This template has an enhancer sequence with two NtrC binding sites, identical to the sequence of the ES-2 duplex, at a distance of about 460 bp from the promoter. In Figure 2 activation of RNAP·σ^{54} transcription by phosphorylated His-tagged NtrC (lane 3) was demonstrated by the appearance of the specific 155 nucleotide transcript. As expected, with the unphosphorylated protein (lane 2) or in the absence of NtrC (lane 1) no transcript was detectable. The experiments shown in Figure 2 were conducted with a NtrC concentration of 15 nM dimer and 5 nM plasmid template. In titrations of our various protein preparations we observed the maximal amount of transcription activation at 15 to 20 nM NtrC. The protein and DNA concentrations were determined by absorbance measurements. This corresponded to a ratio of three to four NtrC dimers per DNA template. Our preparation of His-tagged NtrC was compared to the original *E. coli* wild-type NtrC which had been purified as described

ES-1 DNA duplex (single NtrC binding site)

5'-TGAGATCAGT**TGCACTA**AAA**TGGTGCA**TAATG-3'
3'-ACTCTAGTCA**ACGTGAT**TTT**ACCACGT**ATTAC-5'

ES-2 DNA duplex (two NtrC binding sites)

5'-TCAGT**TGCACTA**AAA**TGGTGCA**TAATGTTAACATTAA**TGCACTA**AAA**TGGTGCA**ACATG-3'
3'-AGTCA**ACGTGAT**TTT**ACCACGT**ATTACAATTGTAATT**ACGTGAT**TTT**ACCACGT**TGTAC-5'

Figure 1. Oligonucleotide duplexes used in this study. The NtrC binding sites are shaded gray and the recognition sequence is printed in bold. The ES-1 duplex (32 bp) has a single binding site and the ES-2 duplex (59 bp) has two NtrC binding sites as indicated.

(Mettke *et al.*, 1995) and was kindly provided by Verena Weiss. No differences were observed between the two protein preparations with respect to DNA binding, transcriptional activation and the association properties as analyzed by analytical ultracentrifugation. This indicates that the His-tag did not alter the properties of the protein.

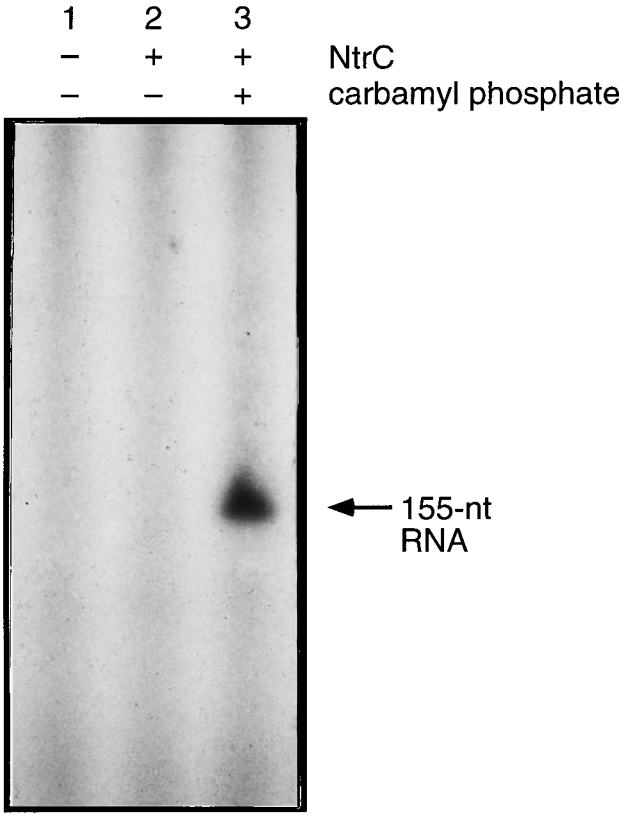

Figure 2. Analysis of the products from the single round transcription assay on a 6% denaturing polyacrylamide gel. Transcription was studied in the absence (lane 1) or presence of NtrC (lane 2 and 3), either unphosphorylated (lane 2) or phosphorylated by the addition of 25 mM carbamyl phosphate (lane 3) as described in Materials and Methods.

Analytical sedimentation equilibrium ultracentrifugation

In order to determine the molecular weight of the different association states of NtrC we used sedimentation equilibrium ultracentrifugation. Typical data sets are displayed in Figure 3, and the results of the analysis are summarized in Table 1. For unphosphorylated NtrC protein in the absence of DNA a molecular weight of 102(±3) kDa was measured in the concentration range of 2 to 8 μM NtrC monomer. This shows that the protein formed a dimer since the calculated M for a monomer is 54 kDa. A data set with the fit to a single exponential (equation (1), Materials and Methods) is displayed in Figure 3a (open circles). The fit was very good and revealed no signs for the presence of additional species, as can be deduced from the residuals of the fit which showed no correlation. Thus, within the concentration range studied (2 to 8 μM NtrC monomer) the only existing species was a dimer.

Typical data for the binding of NtrC to the DNA duplexes ES-1 and ES-2 are presented in Figure 3b. For the analysis of these data a two exponential fit (equation (2), Materials and Methods) was used in which the molecular weight of the free DNA was fixed at 19.9 kDa (ES-1) or 36.6 kDa (ES-2) and only the molecular weight of the complex was allowed to vary. The fit to this model was also very good as can be seen from the residuals shown in Figure 3b. This was also true for the data obtained at other protein concentrations. That the analysis as a non-associating two component system is valid can be explained by the high affinity of NtrC to the DNA and the low absorbance of the free protein at 260 nm as compared to the DNA. We determined a microscopic (or intrinsic) DNA dissociation constant for NtrC of $K_d = 4.6 \times 10^{-10}$ M (Sevenich *et al.*, 1998) under the conditions used for AUC which is consistent with the value given by Weiss *et al.* (1992) if the difference in salt concentration is accounted for. As can be seen in Figure 3b the lowest absorbance in the scans at low radial position is about 0.04 (ES-2)

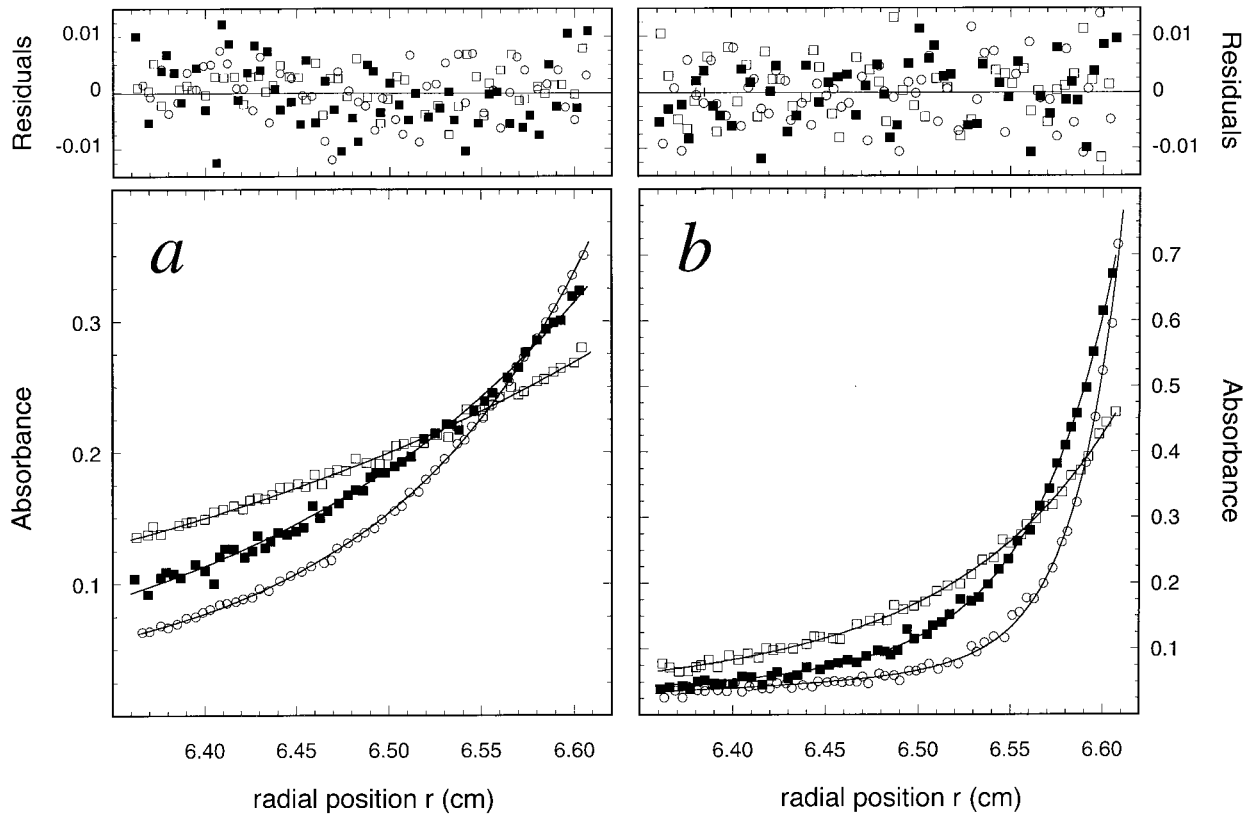

Figure 3. Equilibrium sedimentation data. Centrifugation was carried out at 10,000 rpm and 4°C for 20 hours as described. In the bottom part of the Figure the measured absorbance at 260 nm *versus* the radial position (distance to the center of the rotor) is shown. The top part of the Figure gives the residuals to the fit expressed as the difference between experimental and fitted values. Only every third data point is shown. a, DNA duplex ES-1 (□) at a concentration of 400 nM, DNA duplex ES-2 (■) at a concentration of 200 nM, and NtrC protein (○) at a concentration of 7.2 μM monomer. The continuous line represents the result from a single exponential fit of the data points. For the data presented in this Figure the molecular weights determined from the fit were 21 kDa (ES-1 DNA), 37 kDa (ES-2 DNA), and 107 kDa (NtrC). For average values determined over a range of concentrations see Table 1. b, The continuous line represents the result from the two exponential fit of the data points. In the fit the M of the free DNA component was fixed at 19.9 kDa (ES-1) or at 36.6 kDa (ES-2), and only the M of the DNA-protein complex was allowed to vary. For average values obtained over a range of different protein concentrations see Table 1. (□) NtrC complex formed with 400 nM DNA duplex ES-1 at a protein concentration of about 300 nM dimer. The molecular weight for the complex determined from the fit of this data set was 137 kDa with about 70% of the DNA duplexes being bound by NtrC. (■) NtrC complex formed with 190 nM DNA duplex ES-2 at a protein concentration of about 420 nM dimer. From the fit of the data set shown $M = 262$ kDa was determined for the complex with about 15% of the DNA being free. The residual free ES-2 DNA is likely to be inactive in protein binding due to chemical modification during DNA synthesis (see Materials and Methods). It was not observed with another synthesis of the ES-2 duplex at identical NtrC concentrations. (○) NtrC complex formed upon phosphorylation of the protein by carbamyl phosphate with DNA duplex ES-2 (200 nM) at a protein concentration of about 420 nM dimer. The value of M for the complex determined from the fit was 480 kDa with about 30% of free DNA.

and 0.07 (ES-1) and increases with the distance from the center of the rotor. This absorbance corresponds to a minimal concentration of 80 to 130 nM binding sites, a value which is about 200 times higher than K_d. Thus, throughout the cell we have conditions of stoichiometric binding, i.e. the concentration of free protein is smaller than 1% of the total protein concentration as long as the total protein concentration is below the concentration of binding sites. Under these conditions the absorbance of the free protein can be neglected. After saturation of the binding sites additional protein will be free in the solution. However, since the extinction coefficient of the NtrC dimer at 260 nm is only about 10% of the DNA absorbance of one binding site its contribution can be neglected up to concentrations of free protein that would correspond to that of the DNA. This can be seen most easily in Figure 4. At a constant concentration of 400 nM ES-1 duplex the molecular weight of the complex was determined at protein concentrations from 50 to 900 nM NtrC dimer from a fit of the data to equation (2). The resultant value for M was independent of the protein concentration with an average value of 134(±8) kDa which was very close to that calculated for the complex of one NtrC dimer with ES-1 of 128 kDa (Table 1). Only the points taken at the highest protein concen-

Table 1. Molecular weight (M) of NtrC and DNA complexes determined by analytical ultracentrifugation

	M measured (kDa)	Association state	$\bar{\nu}_{20°C}$ (ml g^{-1})	M calculated (kDa)
DNA duplex ES-1 (single NtrC binding site)	20 ± 2	DNA duplex	0.538	20
DNA duplex ES-2 (two NtrC binding sites)	37 ± 2	DNA duplex	0.538	37
NtrC protein	102 ± 3	NtrC dimer	0.740	108
NtrC protein, ES-1 DNA	134 ± 8	1 NtrC dimer +1 ES-1 duplex	0.709	128
NtrC protein, ES-1 DNA $CH_2NO_5PNa_2$, $MgCl_2$	417 ± 56	Multiple species[a]	0.723[a]	128-513[a]
NtrC protein, ES-2 DNA DNA duplex in excess	161 ± 17[b]	1 NtrC dimer +1 ES-2 duplex	0.689	145
NtrC protein, ES-2 DNA NtrC protein in excess	262 ± 12	2 NtrC dimers +1 ES-2 duplex	0.711	253
NtrC protein, ES-2 DNA, $MgCl_2$	240 ± 8	2 NtrC dimers +1 ES-2 duplex	0.711	253
NtrC protein, ES-2 DNA, $CH_2NO_5PNa_2$	247 ± 7	2 NtrC dimers +1 ES-2 duplex	0.711	253
NtrC protein, ES-2 DNA, $CH_2NO_5PNa_2$, $MgCl_2$,	500 ± 25	4 NtrC dimers +2 ES-2 duplexes	0.711	507
DNA duplex in excess NtrC protein, ES-2 DNA, $CH_2NO_5PNa_2$, $MgCl_2$, NtrC protein in excess	451 ± 16	4 NtrC dimers +1 ES-2 duplex	0.724	470

The data are averages of 5 to 20 measurements and were determined as described in Materials and Methods. For typical data sets see Figure 3. The parameter $\bar{\nu}$ is the calculated partial specific volume of a given complex.

[a] The data indicated the presence of multiple species which according to the sedimentation velocity analysis could include ES-1 with one NtrC dimer (128 kDa), a complex of ES-1 with two NtrC dimers (236-256 kDa), and an octameric complex of NtrC with ES-1 (452-512 kDa). The value of $\bar{\nu}$ refers to a complex of four NtrC dimers and two ES-1 duplexes.

[b] Extrapolation of the data shown in Figure 5 to zero protein concentration after correction for the different partial specific volume.

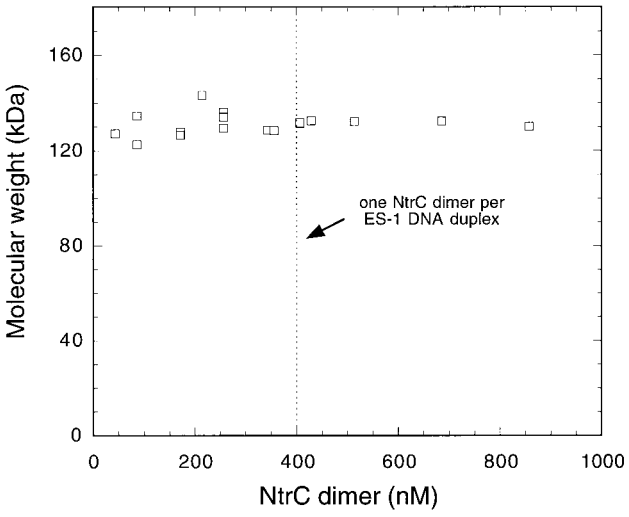

Figure 4. Molecular weight of complexes formed with unphosphorylated NtrC and the ES-1 DNA with a single binding site. The samples included 400 nM ES-1 and the indicated NtrC dimer concentrations in potassium-acetate buffer. The molecular weight of the NtrC·ES-1 complex was determined from a fit of the data to equation (2) with a fixed molecular weight of 19.9 kDa for the free ES-1 DNA duplex.

tration of unphosphorylated protein (800 to 1000 nM in Figures 4 and 5) included a significant contribution of free protein to the measured absorbance. This lead to a small decrease of the average value of M for the complex but the effect was less than the experimental error.

The dependence of the molecular weight of the NtrC-DNA complexes formed with the ES-2 DNA duplex that has two NtrC binding sites is shown over a range of protein concentrations in Figure 5. The open symbols refer to unphosphorylated NtrC. The separate addition of $MgCl_2$ (open circles) or carbamyl phosphate (open triangles) had no significant effect, since the chemical phosphorylation reaction by carbamyl phosphate requires $MgCl_2$ (Feng *et al.*, 1992). At NtrC dimer concentrations below the concentration of binding sites, the apparent molecular weight of the complex increased linearly with the protein concentration (Figure 5). This indicated that binding to the DNA duplex with two binding sites occured with no or very little cooperativity as the data in this regime revealed the existence of a species where only one protein dimer was bound to the DNA. The distribution of the two species (one or two dimers bound) under the conditions of the experiment can be simulated

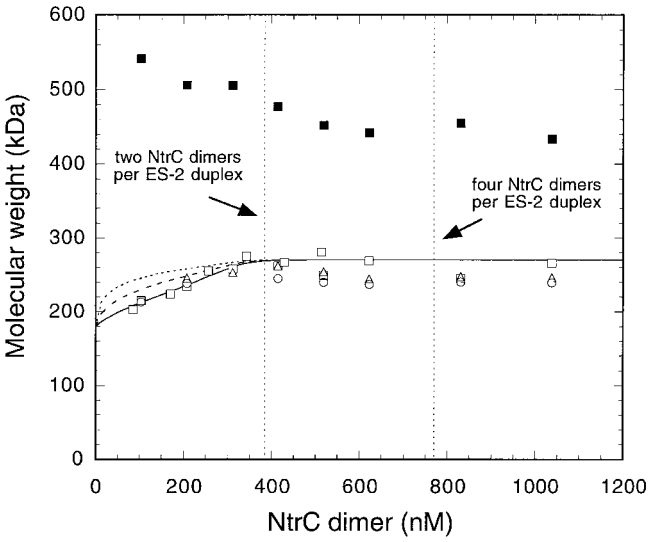

Figure 5. Molecular weight of NtrC complexes at the enhancer DNA sequence ES-2. Complexes were formed with 190 nM DNA duplex ES-2 and the indicated NtrC concentrations either in potassium acetate buffer (□), or in the same buffer supplemented with 10 mM $MgCl_2$ (○), 25 mM carbamyl phosphate (△), or with 10 mM $MgCl_2$ and 25 mM carbamyl phosphate (■). Only the latter conditions will lead to phosphorylation of NtrC. The molecular weight of the NtrC-DNA complex was determined from a two exponential fit of the data. In the fit the molecular weight of the free ES-2 DNA duplex was fixed at 36.6 kDa and only the value of M for the DNA-protein complex was allowed to vary. The results from the analysis and the calculated M for the different association states are summarized in Table 1. The molecular weight data for unphosphorylated NtrC in the absence of $MgCl_2$ and carbamyl phosphate (open squares) were fitted with curves giving the expected average M for different cooperativity constants k_{12} as defined by Ackers *et al*. (1983) namely $k_{12} = 1$ (—), $k_{12} = 5$ (– – –) and $k_{12} = 20$ (· · · · · ·).

with the program BIOEQS (Royer, 1993; Royer & Beechem, 1992) for different cooperativity constants k_{12} as defined by Ackers *et al*. (1983) using the microscopic dissociation constants of $k_1 = k_2 = 5 \times 10^{-10}$ M (Sevenich *et al*., 1998) and a DNA concentration of 190 nM duplex. From these data the average molecular weight of the complex can be determined and curves for $k_{12} = 1$, $k_{12} = 5$ and $k_{12} = 20$ are presented in Figure 5. It can be seen that the best fit of the data is given by the curve for $k_{12} \approx 1$. At saturating protein concentrations the molecular weight became independent of the protein concentration reaching an average value of 250(±12) kDa if all data at saturating protein concentrations were averaged (Table 1). This demonstrates the binding of two NtrC dimers to the two DNA binding sites in agreement with a calculated molecular weight of 253 kDa for this complex.

If NtrC was phosphorylated by the addition of carbamyl phosphate ($CH_2NO_5PNa_2$) and $MgCl_2$ to the buffer its association properties changed dramatically. A specific complex formed at the DNA duplex ES-2 with the enhancer sequence (two NtrC binding sites) that consisted of four NtrC dimers (open circles Figure 3b, and filled squares Figure 5, Table 1). In addition to the two DNA bound NtrC dimers, the two additional NtrC dimers had to be bound *via* protein-protein interactions, because the size of the DNA duplex excludes binding of more than two NtrC dimers to the DNA. The formation of this octameric complex was highly cooperative, since it already appeared at the lowest protein concentrations studied (100 nM NtrC dimer). At this protein concentration an excess of DNA duplexes (total concentration 200 nM) was present. Interestingly, the molecular weight of the complex decreased slowly upon increasing the protein concentration and showed a plateau once saturating protein concentrations were reached. This observation suggests that two DNA strands could bind simultaneously to this complex if an excess of DNA was present (calculated $M = 507$ kDa, Table 1). At saturating protein concentrations the value of M reached a plateau at 451(±16) kDa. This demonstrates that a complex was formed that consisted of four NtrC dimers bound to the ES-2 duplex with a calculated M of 470 kDa (Table 1).

With the ES-1 DNA which has only a single NtrC site the average molecular weight was 417(±56) kDa if NtrC was phosphorylated but the data indicated that more than two species (free ES-1 DNA and its complex with NtrC-P) were present. These could be for example a complex of ES-1 with one NtrC dimer ($M = 128$ kDa), a complex with two NtrC dimers ($M = 236$ to 256 kDa) and a complex with four NtrC dimers (452 to 513 kDa). For the latter two forms the exact value of M will depend on the number of ES-1 duplexes bound. Since the average molecular weight of 417(±56) kDa was only about 10% lower than the value expected for an octameric NtrC complex the data suggest that a significant amount of this association state was present.

Analytical sedimentation velocity ultracentrifugation

In order to confirm the results from the equilibrium runs and to obtain additional information on the shape of the various NtrC and DNA species we conducted a sedimentation velocity analysis. The data were evaluated according to the $g(s^*)$ method developed by Walter Stafford (Stafford, 1992, 1994a, 1997). Since the $g(s^*)$ analysis yields both the sedimentation coefficient s from the peak of the curve and the diffusion constant D from its width, the apparent molecular weight can also be determined (see Materials and Methods). The sedimentation runs are conducted under non-equilibrium conditions and the different species can be partly separated during the runs. For weakly interacting species this could lead to an apparent sedimentation coefficient that is different from that

Table 2. Size and shape parameters of NtrC and DNA complexes determined from sedimentation velocity analysis at 4°C

	$s_{4,buf}$ (S)	$D_{4,buf} \times 10^7$ ($cm^2\ s^{-1}$)	H_2O[a] (g/g)	V_{hyd}[b] (cm^3)	V_{mod}[c] (cm^3)	f/f_o[d]
DNA duplex ES-1 (single NtrC binding site)	1.5 ± 0.1	4.0	0.92	4.8×10^{-20}	5.1×10^{-20}	1.24
DNA duplex ES-2 (two NtrC binding sites)	2.0 ± 0.1	4.1	0.89	8.7×10^{-20}	1.0×10^{-19}	1.45
NtrC protein	2.7 ± 0.1	2.4	0.37	2.0×10^{-19}	2.1×10^{-19}	1.32
NtrC protein, ES-1 DNA	3.3 ± 0.1	2.4	0.45	2.5×10^{-19}	2.6×10^{-19}	1.34
NtrC protein, ES-1 DNA, $CH_2NO_5PNa_2$, $MgCl_2$,	6.5 ± 0.2[e]	4.9	0.42	8.9×10^{-19}	9.2×10^{-19}	1.53
NtrC protein, ES-2 DNA DNA duplex in excess	3.4 ± 0.2[f]	–	0.50	2.8×10^{-19}	3.0×10^{-19}	1.57
NtrC protein, ES-2 DNA NtrC protein in excess	4.7 ± 0.2	1.3	0.45	4.9×10^{-19}	5.1×10^{-19}	1.47
NtrC protein, ES-2 DNA, $CH_2NO_5PNa_2$, $MgCl_2$, DNA duplex in excess	7.5 ± 0.2	3.0	0.45	9.8×10^{-19}	1.0×10^{-18}	1.41
NtrC protein, ES-2 DNA, $CH_2NO_5PNa_2$, $MgCl_2$, NtrC protein in excess	6.3 ± 0.2	1.8	0.41	8.9×10^{-19}	9.2×10^{-19}	1.59

The data are averages of two or three measurements. The standard deviation of s was 3% and for the diffusion coefficient D it was 15%. For typical data sets see Figures 6 and 7. Sedimentation and diffusion coefficients refer to 4°C in KCl buffer and were determined from the $g(s^*)$ plot as described in Materials and Methods (equations (5) to (7)).

[a] Hydration given in g water per g protein or g DNA. The amount of hydration for NtrC was calculated from the amino acid sequence (Kuntz & Kauzmann, 1974) or in the case of the DNA determined from the fit to a cylindrical model (Tirado & Garcia de la Torre, 1979).

[b] V_{hyd} is the hydrated volume calculated from the partial specific volume given in Table 1 and the amount of hydration.

[c] V_{mod} is the volume of the bead model constructed as described in Results. Some of the models are depicted in Figure 8.

[d] Ratio of the measured friction coefficient f to the friction coefficient f_0 of a sphere with the same volume including hydration.

[e] Sedimentation coefficient of the largest peak (60 to 70% of the material) which included at least two different species.

[f] Determined from extrapolation to zero protein concentration.

of a stable complex. However, for hetero-associating systems with an equilibrium dissociation constants of 10^{-9} M and loading concentrations of 100 nM the error in the sedimentation coefficient for the $g(s^*)$ analysis is only in the order of 0.1 S (Stafford, 1994b). In our case K_d is 4.6×10^{-10} M for unphosphorylated NtrC (Sevenich *et al.*, 1998) and 4×10^{-12} M for the cooperative binding of phosphorylated NtrC with $k_{12} = 230$ (Porter *et al.*, 1993). The DNA loading concentrations were at about 600 nM binding sites. With a typical standard deviation of 0.2 to 0.4 S for the determination of the sedimentation coefficients the effect of interacting species can be neglected.

The values of the sedimentation coefficient and the diffusion constant measured at 4°C in buffer are given in Table 2 and the corresponding values corrected to standard conditions (20°C, H_2O) are summarized in Table 3 together with the molecular weight determined from s and D. Representative $g(s^*)$ plots are displayed in Figures 6 and 7. From the measured sedimentation coefficients hydrodynamic models of the different complexes were derived which are shown in Figure 8. Using the program HYDRO (Garcia de la Torre *et al.*, 1994) sedimentation coefficients and diffusion constants were calculated for the different models. These data are included in Table 3 and are compared to the measured values.

In Figure 6a the sedimentation velocity analysis of the two DNA duplexes ES-1 and ES-2 is presented. From the sedimentation coefficient, we determined a hydration of 0.92 and 0.89 g water/g DNA (Table 2). These values were derived from the measured friction coefficient of the DNAs using the model of a circular cylinder (Tirado & Garcia de la Torre, 1979). The dimensions of a cylinder representing the unhydrated DNA were calculated from the partial specific volume of the DNA (0.538 ml g^{-1} at 20°C) and the expected length of 10.9 nm (ES-1) and 20.1 nm (ES-2) according to the number of base-pairs (helical rise 0.34 nm/base-pair). To this cylinder the hydration volume was added by increasing the diameter of the cylinder until the calculated and measured friction coefficients agreed. As an additional constraint the diameter of both duplexes had to be the same, and a value of 2.34 nm for the hydrodynamic diameter of the DNA was computed. The molecular weight of ES-1 and ES-2 determined according to equation (8) gave the expected values (Table 3). Our values of ~30 molecules water/base-pair or 0.9 g water/g DNA hydration (Table 2) and a hydrodynamic DNA radius of 1.17 nm agreed well

Table 3. Sedimentation velocity data of NtrC and DNA complexes compared with hydrodynamic models

	$s_{20,w}$ (S)	s_{model} (S)	$D_{20,w} \times 10^7$ ($cm^2\ s^{-1}$)	$D_{model} \times 10^7$ ($cm^2\ s^{-1}$)	M_{sed}[a] (kDa)	M_{calc} (kDa)
DNA duplex ES-1 (single NtrC binding site)	2.9 ± 0.1	3.0	7.8 ± 1.1	8.0	20 ± 3	20
DNA duplex ES-2 (two NtrC binding sites)	3.9 ± 0.1	3.7	5.9 ± 0.9	5.3	35 ± 6	37
NtrC protein	5.2 ± 0.2	5.3	4.7 ± 0.7	4.6	103 ± 16	108
NtrC protein, ES-1 DNA	6.3 ± 0.2	6.2	4.7 ± 0.7	4.1	112 ± 17	128
NtrC protein, ES-1 DNA, $CH_2NO_5PNa_2$, $MgCl_2$,	12.6 ± 0.4	14.4[b]	9.5 ± 1.3[c]	2.7[b]	408[c]	473[b]
NtrC protein, ES-2 DNA DNA duplex in excess	6.5 ± 0.3[d]	6.4	–	3.4	–	145
NtrC protein, ES-2 DNA NtrC protein in excess	9.0 ± 0.3	9.0	2.6 ± 0.4	3.0	290 ± 46	253
NtrC protein, ES-2 DNA, $CH_2NO_5PNa_2$, $MgCl_2$, DNA duplex in excess	14.4 ± 0.4	15.0	5.8 ± 0.9[c]	2.5	483[c]	507
NtrC protein, ES-2 DNA, $CH_2NO_5PNa_2$, $MgCl_2$, NtrC protein in excess	12.1 ± 0.4	14.4	3.5 ± 0.6[c]	2.7	394[c]	470

The sedimentation and diffusion coefficients given in Table 2 were corrected to standard conditions (20°C, H_2O) according to equations (9) and (10) and are compared to the corresponding values calculated with the program HYDRO for the model complexes shown in Figure 8.

[a] The molecular weight M was determined from s and D according to equation (8).

[b] The calculated values refer to an NtrC octamer with two ES-1 duplexes bound.

[c] The obvious discrepancy in the D values determined from the band broadening in sedimentation velocity analysis and the calculated values for the model can be explained by the simultaneous presence of two or more species which are not resolved in the $g(s^*)$ plot. This will lead to a broadening of the peak and an artificially high apparent value of D. To calculate M from s and D in this case the D values from the model were used with the experimentally determined s values.

[d] Determined from extrapolation to zero protein concentration (see Figure 7a). A diffusion constant could not be determined.

with literature values. Other estimates of DNA hydration have given 22 to 24 molecules water/base-pair that are in direct contact with DNA (Saenger, 1984). Since another 16 to 18 molecules of water/base-pair are believed to be also in the primary hydration shell but not bound directly to the DNA, this would lead to a total of 40 molecules of water/base-pair or 1.16 g water/g DNA (Saenger, 1984). In a recent AUC study the authors estimated a value of 0.8 to 1 g water/g DNA for a set of DNA duplexes (Bonifacio *et al*., 1997).

The $g(s^*)$ plot of NtrC protein (Figure 6b, left curve) showed the presence of a single species with $s_{20,w} = 5.2(\pm 0.2)$ S and $D_{20,w} = 4.7(\pm 0.7) \times 10^{-7}\ cm^2\ s^{-1}$. The value of $M = 103(\pm 16)$ kDa calculated from $s_{20,w}$ and $D_{20,w}$ (Table 3) corresponded to that expected for a dimer (108 kDa) and confirmed the sedimentation equilibrium analysis presented above where $M = 102(\pm 3)$ kDa was determined (Table 1). For the complex of NtrC and ES-1 $s_{20,w} = 6.3(\pm 0.2)$ S and $D_{20,w} = 4.7(\pm 0.7) \times 10^{-7}\ cm^2\ s^{-1}$ were measured (Figure 6b, right curve; Table 3) yielding $M = 112(\pm 17)$ kDa in agreement with the sedimentation equilibrium data ($M = 134(\pm 8)$ kDa; Table 1).

The results of the sedimentation velocity analysis of NtrC complexes formed with the ES-2 DNA fragment support the conclusion that unphosphorylated NtrC does not bind cooperatively to the two binding sites of the ES-2 duplex. This can be seen from the $g(s^*)$ plots at different protein concentrations (Figure 7a, Table 2). At saturating protein concentrations the dominant species had a $s_{4,buf}$ value of 4.7(±0.2) S and no free ES-2 duplex ($s_{4,buf} = 2.0(\pm 0.1)$ S) was detectable (Figure 7a, red curve). The peak at $s_{4,buf} = 4.7(\pm 0.2)$ S represented the species with two NtrC dimers bound (Tables 2 and 3). Upon lowering the protein concentration so that the DNA was in excess (Figure 7a, green and black curves) the $s_{4,buf}$ value shifted to an extrapolated 3.4(±0.2) S at zero protein concentration which was predicted for that of a single NtrC dimer bound to ES-2 (Tables 2 and 3). The intermediate values indicated that a mixture of DNAs with one and two dimers was present. Decomposition of the $g(s^*)$ plot at about 50% free DNA (black curve in Figure 7a) yielded about equal concentrations of the species with one NtrC dimer ($s_{4,buf} = 3.4(\pm 0.2)$ S) and with two dimers ($s_{4,buf} = 4.7(\pm 0.2)$ S). This would correspond to a cooperativity constant of $k_{12} = 1$ (Ackers *et al*., 1983; Hudson & Fried, 1990; Porter *et al*., 1993).

The complexes formed between phosphorylated NtrC and ES-2 displayed again a dependence on the protein/DNA ratio similar to that observed in the equilibrium runs (Figure 5, Tables 2 and 3). At an excess of DNA duplex (Figure 7b, green curve) a peak at $s_{4,buf} = 7.5(\pm 0.2)$ S was observed. Some

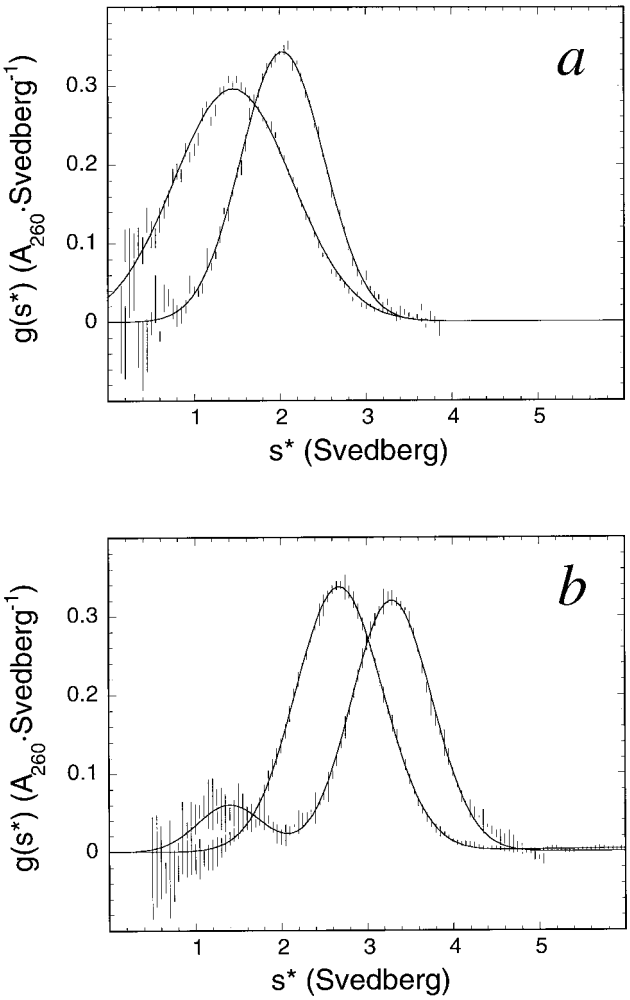

Figure 6. Representative $g(s^*)$ plots of DNA duplexes, free NtrC protein and the NtrC·ES-1 complex. The curves shown are a Gaussian fit of the data. a, DNA duplex ES-1 (left curve) and DNA duplex ES-2 (right curve). b, NtrC protein (left curve) and NtrC·ES-1 complex (right curve). In the latter sample some free ES-1 DNA is present that has a s value of 1.5 S under these conditions (see a).

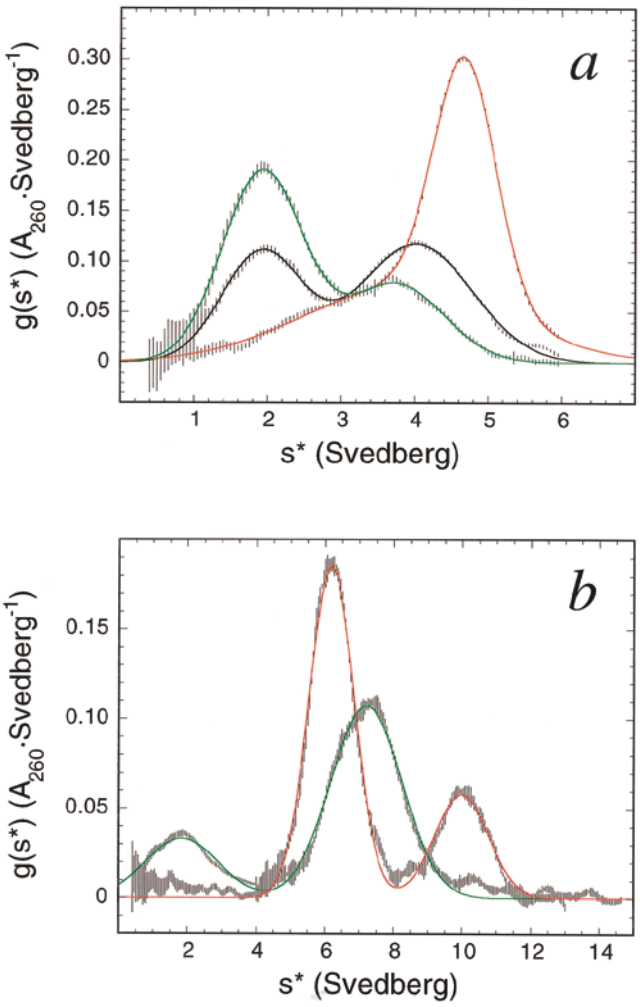

Figure 7. Representative $g(s^*)$ plots of NtrC complexes formed with the ES-2 DNA duplex. The curves dispayed are the fit of the data to a sum of two Gaussian functions. a, DNA duplex ES-2 + NtrC. The DNA duplex concentration was 320 nM (=640 nM binding sites). The curves refer to different protein dimer concentrations: 640 nM (red), 320 nM (black), and 160 nM (green). b, Complexes formed by phosphorylated NtrC with ES-2. The DNA duplex concentration was 320 nM. The two curves refer to different protein dimer concentrations: 1600 nM (red) and 640 nM (green).

free ES-2 DNA ($s_{4,buf} = 2.0(\pm 0.1)$ S) was also present. At increasing protein concentrations the peak at $s_{4,buf} = 7.5(\pm 0.2)$ S shifted to a lower value of $s_{4,buf} = 6.3(\pm 0.2)$ S (note that these are the uncorrected s and D values aquired in KCl buffer at 4°C). At high protein concentrations an additional species appeared with $s_{4,buf} = 10$ S ($s_{20,w} \approx 19$ S, Figure 7b, red curve).

The sedimentation velocity analysis of a sample of phosphorylated NtrC and the ES-1 duplex (data not shown) confirmed the existence of multiple species as already detected in the analysis of the equilibrium runs. From the observed sedimentation coefficients we could identify the free DNA (peak at $s_{4,buf} = 1.5$), the complex of one NtrC dimer and ES-1 (peak at $s_{4,buf} = 3.3$) and a mixture of at least two species (60 to 70% of total material) with a peak at $s_{4,buf} = 6.5$ in the $g(s^*)$ plot (Tables 2 and 3). A complex that includes two NtrC dimers would have a calculated sedimentation coefficient of about 5 S for the conformation shown in Figure 8c and d under the conditions of the experiment. From the results obtained with the ES-2 duplex described above we deduce that the octameric NtrC complex would display $s_{4,buf}$ values of 6 to 8 S. Thus, the data are consistent with the assumption that the majority of the protein associates in complexes that include two to four NtrC dimers. Since the ES-1 DNA is too short to bind more than one dimer per duplex these complexes could be stabilized by the protein-protein interactions depicted in Figure 8c and d.

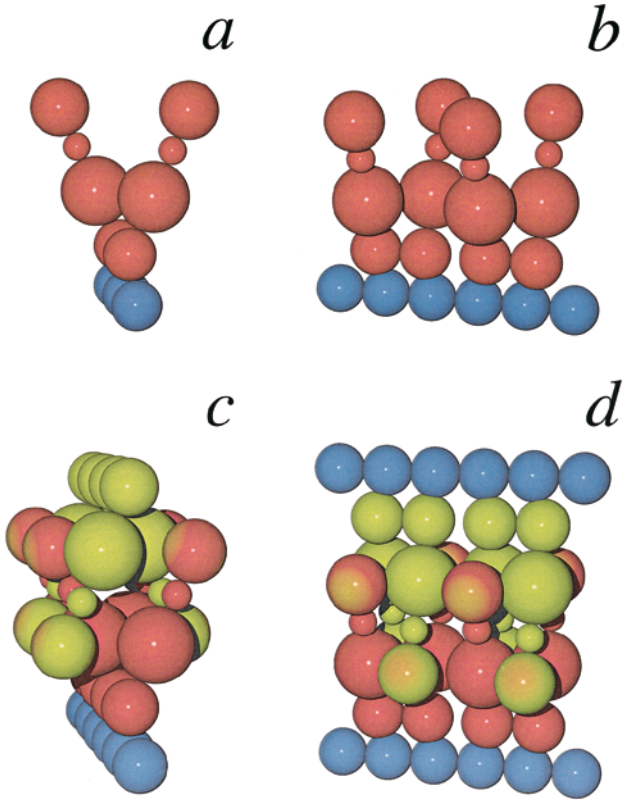

Figure 8. Hydrodynamic models of different NtrC-DNA complexes as derived from sedimentation data. a, ES-1 DNA with one NtrC dimer bound. b, ES-2 DNA with two NtrC dimers bound. c, Complex of four phosphorylated NtrC dimers and one ES-2 DNA. d, Complex of four phosphorylated NtrC dimers and two ES-2 DNAs. The red and yellow spots in c and d indicate phosphorylation of the receiver domain.

Hydrodynamic models

The hydrodynamic properties of DNA are well described with a hydrodynamic diameter of 2.4 nm (Hagerman & Zimm, 1981) which is very close to the hydrodynamic diameter of DNA determined here (2.34 nm) and in the range of 2.1 to 2.7 nm reported previously by various other methods (Bonifacio *et al.*, 1997; Eimer & Pecora, 1991; Kovacic & van Holde, 1977; Porschke, 1991). For a DNA cylinder with a hydrodynamic diameter of 2.4 nm the diffusion constant is reproduced by a string of beads with radius $r = 1.6$ nm (Hagerman & Zimm, 1981; Klenin *et al.*, 1998; Merlitz *et al.*, 1998). Accordingly, our model of the DNA duplex ES-1 consisted of three beads and for ES-2 of six beads with $r = 1.6$ nm. This corresponded to a length of 9.6 nm (ES-1) and 19.2 nm (ES-2), which is close to the calculated length of the two DNAs. Note that only an integral number of beads can be used in the model. The s and D values computed with HYDRO for this parameter set were similar to those measured in the sedimentation velocity runs and demonstrate that the DNA models were adequate (Table 3).

To derive a hydrodynamic model for the NtrC protein from the sedimentation velocity data we used the following approach. From previous studies it has been concluded that the *E. coli* NtrC monomer and the closely related protein from *Salmonella* consists of four domains (Fiedler & Weiss, 1995; North *et al.*, 1996; Osuna *et al.*, 1997): The amino-terminal receiver domain (residues 1 to 120; Volkman *et al.*, 1995), a flexible glutamine rich linker (residues 121 to 133), the central activation domain (residues 134 to 371; Osuna *et al.*, 1997), and the C-terminal DNA binding domain that is homologous to FIS protein (residues 372 to 469; Kostrewa *et al.*, 1991, 1992; North *et al.*, 1993; Pan *et al.*, 1996; Yuan *et al.*, 1991) (note that the *E. coli* NtrC sequence given by North *et al.* (1993) has been revised by Blattner *et al.* (1997)). Each of the domains was modeled as a sphere. The radius r of this sphere was determined from the partial specific volume $\bar{\nu}$ and the amount of hydration as calculated from the amino acid composition (Kuntz & Kauzmann, 1974; Perkins, 1986). The following values were obtained: receiver domain $\bar{\nu} = 0.739$ ml g^{-1}, 0.36 g water/g protein, $r = 1.9$ nm; Q-linker $\bar{\nu} = 0.704$ ml g^{-1}, 0.35 g water/g protein, $r = 0.9$ nm; activator domain $\bar{\nu} = 0.744$ ml g^{-1}, 0.36 g water/g protein, $r = 2.3$ nm; and DNA binding domain $\bar{\nu} = 0.736$ ml g^{-1}, 0.37 g water/g protein, $r = 1.7$ nm (see also Tables 1 and 2 for additional values).

The validity of this approach was checked with bovine serum albumin (BSA) as a reference protein. From the BSA sequence $\bar{\nu} = 0.733$ ml g^{-1} and a hydration of 0.42 g water/g protein were calculated. Since it is known that BSA consists of three domains of approximately the same size we derived a value of $r = 2.1$ nm for the three spheres representing the three BSA domains in agreement with results from the literature (Peters, 1985). The sedimentation coefficients and diffusion constants of BSA computed with HYDRO ($s = 4.5$ S, $D = 6.1 \times 10^{-7}$ cm^2 s^{-1}) agreed very well with the literature values of $s = 4.5$ S and $D = 5.9 \times 10^{-7}$ cm^2 s^{-1} (Peters, 1985). Our measurements reproduced the expected s value with $s = 4.4(\pm 0.1)$ S. The measured D value ($7.4(\pm 1.1) \times 10^{-7}$ cm^2 s^{-1}) was about 20% too high leading to a somewhat too low molecular weight of 55($\pm$8) instead of 67 kDa. This deviation can be explained by the presence of a small amount (<10%) of BSA dimer due to an incomplete reduction of mixed S-S bridges (Peters, 1985) which could lead to a slightly too high D value as determined by the $g(s^*)$ method (see below).

To derive a model of the NtrC dimer we started with the simplest conformation and assumed that the four NtrC domains form a linear array of beads with the above dimensions in the order given by the amino acid sequence, i.e. receiver, Q-linker, activator and DNA binding domain. Since it has been shown that dimerization of NtrC

is mediated by the C-terminal DNA binding domain (Klose *et al*., 1994), we derived the NtrC dimer structure by rotating two monomers which contacted each other at the DNA binding domain until the calculated s and D values matched the measured ones (Table 3). This gave an angle of about 50° between two NtrC monomers and the resulting V shaped model for the NtrC dimer is depicted in Figure 8a and b (red spheres).

Based on the model for the FIS-DNA complex (Pan *et al*., 1996) we constructed the models of NtrC-DNA complexes shown in Figure 8a and b. The $g(s^*)$ plots of these complexes are displayed in Figure 6b (NtrC + ES-1, right curve) and in Figure 7a (NtrC + ES-2). A comparison between the experimental data and those calculated for the models is given in Table 3 and shows an excellent agreement. Since the sedimentation equilibrium analysis indicated that at low protein concentrations a complex of two ES-2 DNAs and four dimers of phosphorylated NtrC was formed and at saturating protein concentrations only one ES-2 duplex was present in the complex (Figure 5), we devised the models shown in Figure 8d and c for the two species. The calculated sedimentation coefficients $s_{20,w}$ for the models were in good agreement with the measured values as shown in Table 3: 14.4(±0.4) S *versus* 15.0 S for the conformation with two DNA strands bound (Figure 8d) and 12.1(±0.4) S *versus* 14.4 S for the octameric complex with one ES-2 duplex (Figure 8c). The obvious discrepancy in the value of D calculated from the band broadening in the sedimentation velocity analysis can be explained by sample inhomogeneity. Any unresolved mixture of two or more species will lead to an artificially high apparent value of D and will yield a molecular weight as calculated according to equation (8) that is too low.

Discussion

Activator binding at the enhancer DNA sequence has important consequences: the DNA linkage between activator protein at the enhancer and the transcription machinery at the promoter can potentiate interactions that would not occur if the proteins were free in solution by raising the local concentration of activator in the vicinity of the promoter (Merlitz *et al*., 1998; Rippe *et al*., 1995). Furthermore, enhancers can facilitate the assembly of a specific multimeric activator complex, thus giving this complex a significant equilibrium advantage over the concentration at which it is present free in solution. This idea is consistent with the observation that enhancers often contain multiple protein binding sites that act synergistically. Thus, it is important for the understanding of the transcription process to examine the activating species that forms at the enhancer. To this end we have studied the DNA binding and multimerization of NtrC by analytical ultracentrifugation (AUC).

Association states of unphosphorylated NtrC

In previous studies it was concluded from gel filtration and gel electrophoresis data that unphosphorylated NtrC forms a dimer and binds as a dimer to a single site (Klose *et al*., 1994; Porter *et al*., 1993; Reitzer & Magasanik, 1983). These results were confirmed in our studies by AUC which provides a more accurate and reliable determination of the molecular weight at defined conditions and at equilibrium. For protein concentrations in the range studied (2 to 8 μM NtrC monomer) within the accuracy of our determination the only existing species was a dimer, i.e. the dimer dissociation constant K_d was significantly smaller than 1 μM. In a study of NtrC from *Klebsiella pneumoniae* (Farez-Vidal *et al*., 1996) the author determined a K_d of 7 μM for the monomer-dimer equilibrium by AUC at comparable salt concentrations. Our results demonstrate a significantly stronger interaction between NtrC monomers with the *E. coli* protein studied here.

In the range of protein concentrations used for DNA binding (50 to 1000 nM NtrC dimer) the unphosphorylated protein was bound as a dimer to a single binding site. In addition, our data indicate that at non-saturating protein concentrations also complexes formed at the DNA with two binding sites where only one of the two binding sites was occupied. From an estimate of the relative amount of this species in both the sedimentation equilibrium and the sedimentation velocity runs we deduced that binding of unphosphorylated NtrC to the ES-2 duplex occured essentially without cooperativity. The same result was obtained in fluorescence anisotropy measurements of NtrC binding to the same DNA fragment (Sevenich *et al*., 1998). Furthermore in a study of NtrC oligomerization by scanning force microscopy the authors determined at a DNA concentration of 3.3 nM and an excess of protein that 72% of the complexes had two dimers bound and 22% had one dimer bound (Wyman *et al*., 1997), which would be inconsistent with a positive cooperativity of binding. However, in the literature it has also been reported that binding of two unphosphorylated NtrC dimers to the enhancer occurs with a cooperativity constant of 20 to 100, as determined by gel-shift experiments, nitrocellulose filter binding assays and quantitative DNA footprinting (Chen & Reitzer, 1995; Porter *et al*., 1993; Weiss *et al*., 1992). For the discussion of these data one important parameter is the effect of ATP on NtrC binding. In the presence of 2 mM ATP a cooperativity constant of 20(±6) (Porter *et al*., 1993), 37 (Weiss *et al*., 1992) and 100 (Chen & Reitzer, 1995) was measured. In the absence of ATP the cooperativity constant decreased from 100 to 20 (Chen & Reitzer, 1995). The values obtained by Weiss *et al*. (1992) and Chen & Reitzer (1995) were determined for a somewhat different sequence of NtrC binding sites as compared to the ES-2 duplex studied here which is identical to that analyzed by Porter *et al*. (1993). The latter study

has also the advantage that the cooperativity constant has been determined in a way that was independent of the protein concentration, which excludes a possible source of errors. Since a cooperativity constant of 20(±6) has been determined in the presence of ATP (Porter *et al.*, 1993) it appears reasonable to assume that the corresponding value in the absence of ATP would be significantly lower and in the range of the value determined here. In addition, the results from the fluorescence anisotropy measurements (Sevenich *et al.*, 1998) and the analysis by scanning force microscopy (Wyman *et al.*, 1997) mentioned above were also conducted in the absence of ATP and thus support this conclusion.

For the unphosphorylated NtrC-DNA complexes the agreement between the measured *s* and *D* values and those calculated with HYDRO were excellent (Table 3). In addition, the value of *M* determined from the sedimentation runs was equivalent to that determined in the equilibrium analysis but with a somewhat larger standard deviation (Tables 1 and 3). Thus, the models derived for the NtrC dimer and the protein-DNA complexes shown in Figure 8a and b fit the experimental results exactly. Obviously, other arrangements of the different NtrC domains are possible that would also lead to a good fit with the AUC data obtained for the unphosphorylated protein. However, the V shaped model for the NtrC dimer conformation presented here has the additional advantage that it can be easily extended to a model for the octameric association states adopted by the phosphorylated protein as shown in Figure 8c and d.

Association states of phosphorylated NtrC

In vivo phosphorylation is brought about by the histidine kinase NtrB (see Fiedler & Weiss, 1995, and references therein). This phosphorylation can be reproduced *in vitro* by the addition of a phosphorylating chemical like carbamyl phosphate (Feng *et al.*, 1992). In studies of the degree of phosphorylation of NtrC by NtrB and ATP it was found that the maximal degree of phosphorylation of NtrC is only 35% at 37°C due to the autophosphatase activity of NtrC (Weiss & Magasanik, 1988). Although this autophosphatase activity is reduced at 4°C, the temperature at which the analytical ultracentrifugation experiments were conducted, it appears unlikely that with carbamyl

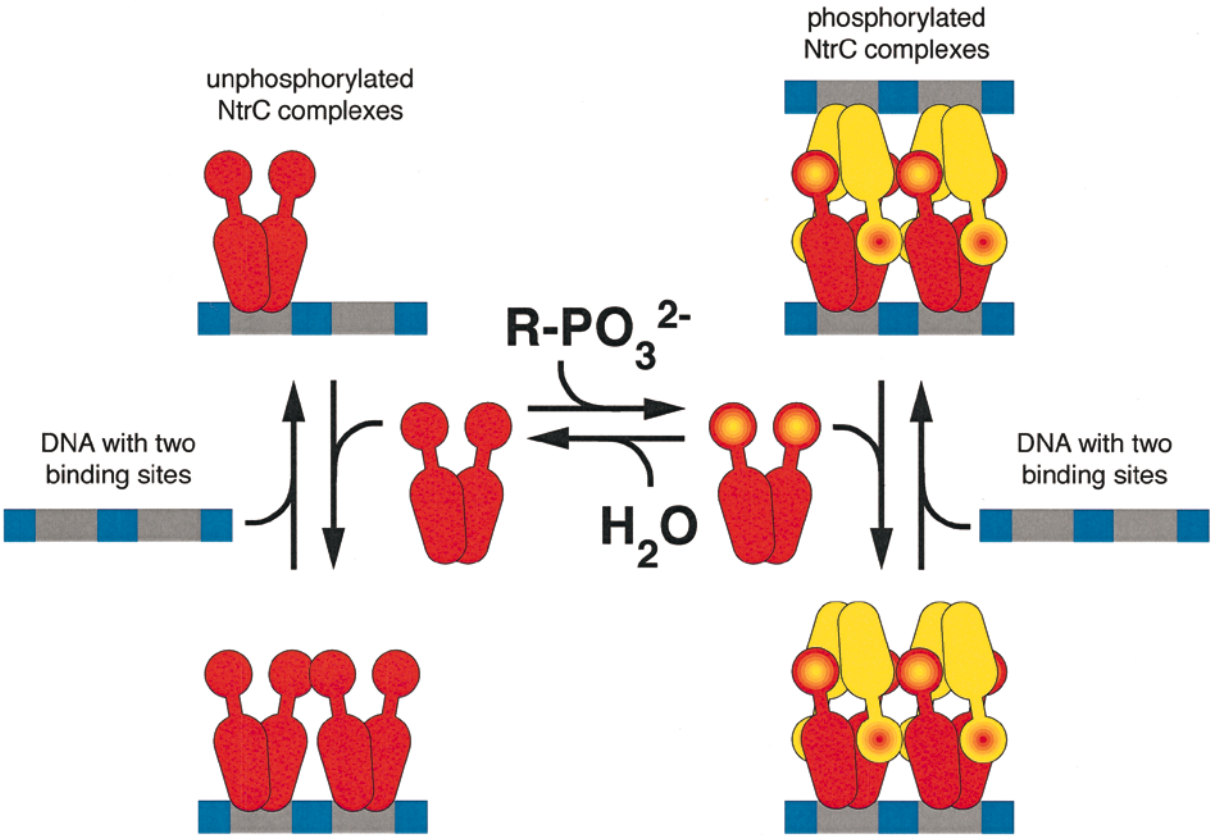

Figure 9. Different association states of NtrC formed at the enhancer. The NtrC complexes that have been observed by AUC with the ES-2 DNA duplex are shown. The ES-2 DNA duplex sequence is given in Figure 1 and has two binding sites for NtrC indicated by the gray shading. NtrC dimers are drawn in red and in yellow. Phosphorylation of the NtrC receiver domain is shown by yellow or red spots in the receiver domain. The phosphoryl group ($R\text{-}PO_3^{2-}$) is either added by a specific kinase NtrB from ATP or by phosphorylation *via* a small molecule phosphodonor like carbamyl phosphate which has been used here.

phosphate as a phosphate donor a higher degree of phosphorylation is achieved because NtrB is a more effective phosphate donor (Lukat *et al*., 1992). Thus, we estimate that only a quarter to a third of the NtrC monomers are phosphorylated in equilibrium by carbamyl phosphate in our samples. Accordingly, the formation of the octameric NtrC complex has to be compatible with an incomplete phosphorylation of the protein. We would like to note that in the model of the octameric complex (Figure 8c and d) it would be enough if one or two NtrC monomers (i.e. a degree of 13 to 25% phosphorylation) are phosphorylated to stabilize the complex by interaction between two different NtrC dimers.

The results of the AUC experiments with NtrC and the two DNA duplexes described here lead to the scheme shown in Figure 9. In addition to the two DNA bound NtrC dimers that are depicted in red, two additional NtrC dimers (yellow) are bound *via* protein-protein interactions if the protein is phosphorylated. The observed decrease of the molecular weight of the complex upon increasing the protein concentration suggests that two DNA strands can bind simultaneously to this complex if an excess of DNA is present (Figure 8d). At increasing protein concentration the equilibrium is shifted to the species with only one DNA duplex bound (Figure 8c). The molecular weight determined in the equilibrium runs was 500(±25) and 451(±16) kDa for the two species. This compares favorably to the calculated molecular weight of 507 and 470 kDa (Table 1).

The results obtained in the sedimentation equilibrium runs were fully consistent with the sedimentation velocity analysis. The calculated sedimentation coefficients $s_{20,w}$ for the models agreed well with the experimental data (Table 3). The measured values of 14.4(±0.4) S for the sample with DNA excess and 12.1(±0.4) S for the sample with protein excess displayed the expected shift of the sedimentation coefficient if one or two DNA strands were bound. The fit between the experimental data and the model was not as good as with the unphosphorylated complexes. However, it appears likely that some conformation changes of the NtrC dimer occur in the phosphorylated octameric complex as compared to the free unphosphorylated dimer. Taken into account that no such modification of the NtrC dimer conformation has been introduced to the model the observed agreement is quite good and strongly supports the model.

The obvious discrepancy in the value of D calculated from the band broadening in the sedimentation velocity analysis for the phosphorylated NtrC-DNA complexes (Table 3) can be explained by the co-existence of the two slightly different octameric complexes. If two species are present that are not well separated, this will lead to a broadening of the peak in the $g(s^*)$ plot and an artificially high apparent value of D. This in turn will result in a calculated value for M that is too low. A careful inspection of the Gaussian fit curve reveals that indeed the fit to two species in Figure 7b was not as good as for the other complexes (Figures 6 and 7a). This is to be expected if the peaks represent two species that are not resolved. Thus, it would be most consistent with the data to assume that both the peak at $s_{4,buf} = 7.5(\pm 0.2)$ S (Figure 7b, green curve) and the one at $s_{4,buf} = 6.3(\pm 0.2)$ S (Figure 7b, red curve) represent a mixture of the octameric NtrC complexes with one or two DNAs bound as displayed in Figure 8c and d.

At very high protein concentrations (Figure 7b, red curve) an additional species with $s_{4,buf} \approx 10$ S ($s_{20,w} \approx 19$ S) appeared. This species could be a dimer of two octameric complexes ($M \approx 1000$ kDa). It would hardly be detectable in the equilibrium runs, since it would sediment to the bottom of the cell under the conditions used. Indeed we observed some loss of material (15 to 20%) at the highest protein concentrations as deduced from a comparison of the integrated absorbance of the first scan taken at 3000 rpm and the scans taken after centrifugation for 18 to 22 hours when an equilibrium had been established. It appears questionable whether the 19 S NtrC complex is of biological relevance, because the required protein concentrations are well above those found in the bacterial cell which are estimated to be 10 to up to 100 nM under nitrogen limiting conditions (Reitzer & Magasanik, 1983). It is possible that *in vivo* the 19 S species could already form at these relatively low protein concentrations. However, a specific biological function of this association state seems unlikely since all known biological activities of NtrC like transcriptional activation, ATPase activity and DNA binding can be reproduced *in vitro* at nanomolar concentrations.

In a previous study it has been shown that transcription activation at high NtrC-P concentration also occurs from a template with only a single NtrC binding site (Weiss *et al*., 1992). To address this point we have analyzed the complexes formed with NtrC-P and the ES-1 duplex, which has only a single NtrC binding site. As mentioned above our results indicate that a mixture of several species was formed with ES-1 and phosphorylated NtrC which appeared to include significant amounts of an octameric NtrC complex but also other species like for example a complex of two NtrC dimers. Thus, the data suggest that the octameric complex can also form with the ES-1 DNA albeit less efficiently as compared to the ES-2 duplex. This would be in complete agreement with the transcription experiments of Weiss *et al*. (1992).

Implications of the model for the phosphorylated NtrC complex

It is known that a single phosphorylated NtrC dimer is not able to activate transcription and that phosphorylation induces the ATPase activity of NtrC (Austin & Dixon, 1992; Mettke *et al*., 1995;

Porter *et al.*, 1993; Weiss *et al.*, 1991, 1992; Wyman *et al.*, 1997). From the results presented here we conclude that activation occurs *via* the following mechanism: Upon phosphorylation NtrC assembles to a specific octameric complex at the enhancer DNA sequence that consists of four NtrC dimers and has an ATPase activity. This complex does not form with unphosphorylated NtrC. The octameric NtrC-P complex has a unique ability to interact with the promoter bound RNAP·σ^{54} holoenzyme to catalyze the melting of the promoter DNA. This in turn leads to the initiation of transcription. It is tempting to speculate that the octameric NtrC-P complex can make multiple contacts with RNAP·σ^{54} that are not possible if two dimers of unphosphorylated protein are present. This could lead to an increased affinity for RNAP·σ^{54} and a coupling of ATP hydrolysis with open complex formation. Experiments are in progress to determine the affinity of the different NtrC association states to the polymerase complex, in order to provide further details on the activation mechanism by which NtrC operates.

As discussed above we have derived a hydrodynamic model for the octameric complex of NtrC-P that makes several predictions which we hope will be tested in future experiments. In particular we would like to note the following. According to our model, the octameric complex of phosphorylated NtrC is able to bind two DNA strands. Thus, it may be possible for the DNA to wrap around the NtrC complex at the enhancer which is consistent with the results from electron microscopy and scanning force microscopy studies (Brahms *et al.*, 1995; Rippe *et al.*, 1997; Wyman *et al.*, 1997). This might be important for stabilizing a specific DNA conformation and/or binding to superhelical DNA with curved inserts as observed previously (Brahms *et al.*, 1995). Furthermore, the model presented in Figure 8 suggests that interactions occur between the phosphorylated receiver domain and the central activation domain of different NtrC dimers. Such an interaction could be the reason for the observed strong cooperativity of DNA binding and complex formation upon phosphorylation. It provides a simple explanation on how a conformation change in the receiver domain induced by phosphorylation is transmitted to the central domain in which the transcriptional activation function resides. Since both domains are connected *via* a flexible linker it appears unlikely that phosphorylation of the receiver domain would change the conformation of the activation domain in the same NtrC monomer. In contrast, for the spatial arrangement in the phosphorylated NtrC complex suggested here the interaction between a receiver domain and one or even two activation domains of different NtrC dimers is easily possible. In a study of constitutive NtrC forms transcriptional activation and ATP hydrolysis were analyzed *in vitro* for the ΔN-NtrC^{S160F} mutant which is lacking the N-terminal receiver domain and has a point mutation in the central activation domain (Flashner *et al.*, 1995). The authors concluded that oligomerization is mediated for ΔN-NtrC^{S160F} solely by the central activation domain and not by an interaction between the phosphorylated receiver domain and the activation domain as proposed in our model. However, it is possible that an artifactual protein-protein interaction is created by the S160F point mutation in the activation domain and/or by the deletion of the amino-terminal domain. A comparison of the data presented here with studies of NtrC^{S160F} (Farez-Vidal *et al.*, 1996; Sevenich *et al.*, 1998) indicate that the association properties of the NtrC^{S160F} mutant and the phosphorylated wild-type protein are rather different. Thus, it remains to be studied in further detail if oligomerization proceeds by the same mechanism.

Concluding Remarks

Analytical ultracentrifugation is by no means a new technique. It has been used successfully since the 1950s in studies of biological macromolecules. However, as has been pointed out in a number of recent reviews (Hensley, 1996; Schuster & Toedt, 1996; Stafford, 1997), the availability of new instruments like the Beckman XL-A and improved methods for data analysis make the use of AUC both much faster and easier. In particular, we would like to emphasize that the program DCDT by Walter Stafford (Stafford, 1992, 1994a, 1997) and the program HYDRO developed by José Garcia de la Torre and co-workers (Garcia de la Torre, 1989, 1992; Garcia de la Torre & Bloomfield, 1981; Garcia de la Torre *et al.*, 1994) have been extremely helpful for the analysis of the data presented here.

As compared to the standard molecular biological techniques the use of AUC offers a variety of advantages. The molecular weight can be determined with high accuracy at thermodynamic equilibrium and different species can be resolved. The experiments are conducted in biological buffer solutions so that the sample is in the native and fully hydrated state and information on the biologically active conformation can be obtained. These features are crucial for a more detailed and quantitative analysis of multimeric protein-DNA complexes which appear to exert central functions in the transcription regulation process.

Materials and Methods

DNA purification

DNA oligonucleotides purified by reversed phase HPLC were purchased from MWG Biotech (Ebersberg, Germany). The extinction coefficient of the single strands was determined as described previously (Rentzeperis *et al.*, 1992; Sevenich *et al.*, 1998) and values of $\varepsilon_{260} = 455{,}000\ M^{-1}\ cm^{-1}$ (ES-1) and $\varepsilon_{260} = 840{,}000\ M^{-1}\ cm^{-1}$ (ES-2) were derived. The DNA duplexes were purified on a 12% (w/v) non-denaturing polyacrylamide gel, by cutting out the duplex band and extracting it from the gel as described (Maniatis *et al.*, 1982). Traces of acryl-

amide and other impurities were removed with the Qiaex II gel extraction kit from Qiagen (Hilden, Germany).

One batch of the purified synthetic ES-2 duplexes showed a residual amount of 10 to 15% free DNA duplex being present even in the presence of excess of NtrC protein at concentrations well above the dissociation constant for NtrC binding. The ES-2 DNA purified from the other two syntheses was fully active under identical conditions (>95% DNA bound). Partial inactivity of DNA is sometimes found with synthetic DNA fragments and is likely to be related to chemical modifications introduced during the synthesis to a small percentage of the strands (Carey, 1991). It should not effect the molecular weight determinations in the equilibrium runs and sedimentation velocity experiments since the presence of free DNA duplex was accounted for in the data analysis as described below.

Plasmid construction and protein purification

The protein expression vector pNTRC-3 was created to permit over-expression of His-tagged wild-type NtrC and was constructed as follows. The NtrC gene from *E. coli* missing the first 14 amino acids was cut out with the restriction endonucleases *Van*91I and *Bam*HI from plasmid pFI20. Plasmid pFI20 is a derivative of pRH800 (Lange & Hengge-Aronis, 1994) and was a kind gift from Verena Weiss and Ulrike Fiedler, University of Konstanz. The resulting fragment was ligated to an oligonucleotide duplex to add the missing 14 amino acids from the N terminus and to create a *Nde*I site. The resulting *Nde*I-*Bam*HI fragment with the NtrC gene was then ligated into the same sites in pET15b to construct plasmid pNTRC-3. From this vector wild-type *E. coli* NtrC was expressed with an additional N-terminal His-tag having the sequence MGSSHHHHHHSSGLVPRGS, which was used for affinity purification on Ni-chelating resins. Accordingly, the His-tagged protein used in this study had a slightly higher molecular weight (54 kDa) than the wild-type protein which has a molecular weight of 52 kDa, as calculated from the amino acid composition. The sequence of the NtrC gene in pNTRC-3 was confirmed by DNA sequencing. Expression and purification of wild-type NtrC protein from pNTRC-3 was done according to the same procedure that has been used to purify the His-tagged mutant protein $NtrC^{D54E,S160F}$ (Rippe *et al*., 1997). The purified protein stock was in 20 mM Tris-HCl (pH 7.9), 180 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 5% glycerol (v/v) and was frozen in liquid nitrogen before being stored at −80°C. The extinction coefficient of NtrC was calculated from the amino acid sequence to be $\varepsilon_{280} = 46{,}400\ M^{-1}\ cm^{-1}$ (monomer) and $\varepsilon_{280} = 92{,}800\ M^{-1}\ cm^{-1}$ (dimer) (Gill & von Hippel, 1989). From the absorbance spectrum of the protein we determined the corresponding values at 260 nm to be $\varepsilon_{260} = 27{,}300\ M^{-1}\ cm^{-1}$ (monomer) and $\varepsilon_{280} = 54{,}600\ M^{-1}\ cm^{-1}$ (dimer).

BSA (electrophoresis grade and fatty acid free) as a reference protein for the sedimentation velocity analysis was bought from Sigma. It was dialyzed against 10 mM Tris-HCl (pH 6.8), 100 mM KCl, 1 mM DTT overnight. For the AUC experiments the 10 mg/ml BSA stock solution was diluted 20-fold into KCl buffer (see below).

Transcription reactions

Single round transcription assays (Figure 2) were performed by a procedure similar to that described by Klose *et al*. (1993). $RNAP \cdot \sigma^{54}$ at a concentration of 50 nM, and NtrC at 15 nM dimer either unphosphorylated or phosphorylated by the addition of 25 mM carbamyl phosphate, were incubated with 5 nM of the DNA template pJES534 (Klose *et al*., 1993; Porter *et al*., 1993) in a buffer containing 40 mM Hepes-KOH (pH 8.0), 10 mM magnesium acetate, 100 mM potassium acetate, 100 μg/ml BSA and 1 mM DTT for ten minutes at 37°C to allow protein binding. Open complex formation was initiated by adding ATP to a concentration of 5 mM and the samples were incubated for another ten minutes. Then a mixture of GTP (to 1 mM), CTP/[α-^{32}P]CTP (to 25 μM) and heparin (to 100 μg/ml) was added to permit for a single transcription cycle. After ten minutes the reaction was stopped by addition of formamide gel loading solution and the radioactive transcripts were analyzed on a 6% denaturing polyacrylamide gel.

Analytical sedimentation equilibrium ultracentrifugation

Analytical sedimentation equilibrium ultracentrifugation was carried out on a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics and an An60Ti rotor. NtrC protein and DNA duplexes ES-1 or ES-2 were mixed in a buffer containing 20 mM Tris-HCl (pH 7.9), 180 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, and 5% glycerol (KCl buffer). Some measurements were made also in 10 mM Tris-acetate (pH 8.0), 150 mM pottassium acetate, 0.1 mM DTT, 0.1 mM EDTA, 5% glycerol (potassium acetate buffer), but no difference between the buffers was observed. As indicated, the buffer was supplemented with 25 mM carbamyl phosphate and/or 10 mM $MgCl_2$. The DNA duplex concentration was usually kept around 400 nM (ES-1) or 200 nM (ES-2) which is equivalent to an absorbance of 0.2 at 260 nm and 12 mm path length. Centrifugation was carried out at 4°C and at 10000 rpm and for some runs also at 5000 rpm until equilibrium was reached (24 to 48 hours). The analysis of the data obtained at 10,000 or 5000 rpm showed no dependence of the molecular weight on the rotor speed within the error of the measurements. For the determination of the molecular weight the data were analyzed with the software provided by Beckman which is based on the program Origin v.3.78 (MicroCal Software, Inc. MA) and performs either a single exponential (model ideal1, equation (1)) or a two exponential fit (model ideal2, equation (2)) to the data:

$$A_r = A_0 \cdot \exp\left(\frac{M \times (1 - \bar{v} \times \rho) \times \omega^2 \times (r^2 - r_0^2)}{2 \times RT}\right) + E \quad (1)$$

$$A_r = A_{0,1} \times \exp\left(\frac{M_1 \times (1 - \bar{v}_1 \times \rho) \times \omega^2 \times (r^2 - r_0^2)}{2 \times RT}\right) + A_{0,2} \times \exp\left(\frac{M_2 \times (1 - \bar{v}_2 \times \rho) \times \omega^2 \times (r^2 - r_0^2)}{2 \times RT}\right) + E \quad (2)$$

In equations (1) and (2) A_r and A_0 are the absorbance at a radial position r and at a reference position r_0. Thus, the absolute concentrations or extinction coefficients do not enter these equations and need not be known for the determination of the molecular weight M. It is only required that the absorbance of a given component is proportional to the concentration which is true as long as the Beer-Lambert relation is true ($A_r < 1.2$ to 1.5). The

indices 1 and 2 in equation (2) correspond to species 1 and 2, respectively. The angular velocity ω is known from the rotor speed, R is the universal gas constant, and T is the absolute temperature. The parameter E is the baseline offset and was determined from the absorbance of the region close to the meniscus after sedimenting the protein sample at 48,000 rpm for six hours. The parameter M is the molecular weight of the macromolecule studied which was derived from the fit, and $\bar{\nu}$ is the partial specific volume. For the His-tagged NtrC studied here it was calculated from the amino acid composition and corrected for the temperature of the experiment to be 0.733 ml g^{-1} at 4°C or 0.740 ml g^{-1} at 20°C (Laue *et al.*, 1992; Perkins, 1986). For the DNA duplexes a value of 0.531 ml g^{-1} at 4°C (=0.538 ml g^{-1} at 20°C) was taken. This value was chosen instead of the average value of 0.55 ml g^{-1} for nucleic acids (Durschlag, 1986) to give exactly the calculated molecular weight of the DNA duplexes in the equilibrium runs used under our specific experimental conditions. For short duplexes it has been shown that the partial specific volume displays some dependence on the sequence (Bonifacio *et al.*, 1997). The values for $\bar{\nu}$ at 20°C of the different species are given in Table 1. The temperature correction to 4°C and 20°C of the calculated partial specific volume $\bar{\nu}$ at $T = 298$ K were made with equation (3) (Durschlag, 1986):

$$\bar{v}_T = \bar{v}_{25} + [4.25 \times 10^{-4} \times (T - 298.15)] \quad (3)$$

The density ρ of the buffer was measured to be 1.024 g ml^{-1} (KCl buffer) and 1.021 g ml^{-1} (K-acetate buffer) at 4°C with a DMA 5000 density meter (Anton Paar, Graz, Austria).

Analytical sedimentation velocity ultracentrifugation

Analytical sedimentation velocity studies were done in KCl buffer (see equilibrium runs). The DNA concentration was kept at 640 nM ES-1 and 320 nM ES-2 duplex ($A_{260} \approx 0.3$). Centrifugation was carried out at 4°C and at 42,000 rpm in epon double-sector cells. Data were collected at 260 nm except for the pure protein sample which was measured at 280 nm using a spacing of 0.01 cm with four averages in a continuous scan mode. The velocity data were analyzed with the program DCDT (Stafford, 1992, 1994a, 1997). The scans to be analyzed were selected from the end of the run and the time window Δt to be analyzed was estimated as suggested in the DCDT manual according to equation (4):

$$\Delta t < \frac{1.6 \times 10^5 \times t}{\sqrt{M} \times \text{speed}} \quad (4)$$

In equation (4) the molecular weight M is given in kDa and the rotor speed is given in rpm. The program DCDT generates a distribution of apparent sedimentation coefficients $g(s^*)$ by taking the differences in absorbance profiles of scans according to equation (5):

$$g(s^*) = \left(\frac{\partial c}{\partial t}\right)_{\text{corr}} \cdot \left(\frac{1}{c_0}\right) \cdot \left(\frac{\omega^2 \cdot t^2}{\ln(r/r_m)}\right) \cdot \left(\frac{r}{r_m}\right)^2 \quad (5)$$

The method is described and discussed in detail in the literature (Stafford, 1992, 1994a, 1997). The resulting $g(s^*)$ *versus* s^* curve is essentially of Gaussian shape and can be fitted to equation (6) to determine the apparent sedimentation coefficient s from the maximum of the curve and the standard deviation σ as described by Stafford (1997):

$$g(s^*) = A \times \exp\left(-\frac{(s^* - s)^2}{2 \times \sigma^2}\right) \quad (6)$$

The standard deviation σ of the fit curve is then used to determine the diffusion coefficient D from equation (7):

$$D = \frac{(\sigma \times r_m \times \omega^2 \times t)^2}{2 \times t} \quad (7)$$

From s and D the molecular weight is calculated:

$$M = \frac{s}{D} \times \frac{RT}{(1 - \bar{v} \times \rho)} \quad (8)$$

The experimentally determined sedimentation coefficients s_{exp} and diffusion coefficients D_{exp} measured at 4°C in buffer were corrected to the standard conditions (20°C, H_2O) by using the equations below (Cantor & Schimmel, 1980):

$$\frac{s_{20,w}}{s_{\text{exp}}} = \left(\frac{1 - \bar{v}_{20,\text{w}} \times \rho_{20,w}}{1 - \bar{v}_{T_{\text{exp}},\text{buf}} \times \rho_{T_{\text{exp}},\text{buf}}}\right) \times \left(\frac{\eta_{T_{\text{exp}},w}}{\eta_{20,w}}\right) \times \left(\frac{\eta_{T_{\text{ref}},\text{buf}}}{\eta_{T_{\text{ref}},w}}\right) \quad (9)$$

$$\frac{D_{20,w}}{D_{\text{exp}}} = \left(\frac{293\,\text{K}}{T_{\text{exp}}}\right) \times \left(\frac{\eta_{T_{\text{exp}},w}}{\eta_{20,w}}\right) \times \left(\frac{\eta_{T_{\text{ref}},\text{buf}}}{\eta_{T_{\text{ref}},w}}\right) \quad (10)$$

In these equations η is the viscosity. The index T_{exp} refers to the experimental temperature, T_{ref} to a common reference temperature and *buf* to the buffer solution used. The density of water at 20°C is $\rho_{20,\text{w}} = 0.9982$ g ml^{-1}. The viscosity at this temperature $\eta_{20,\text{w}}$ is 1.002 mPa second and at 4°C $\eta_{4,\text{w}} = 1.567$ mPa second (Lide, 1996). For the KCl buffer at 20°C the viscosity was measured to be $\eta_{20,\text{KCl}} = 1.182$ mPa second using a Schott KPG Ubbelohde capillary viscometer with automatic sampler (Schott, Hofheim, Germany).

Modeling

Different models for NtrC and its complexes with DNA were constructed as described in Results. For these models the sedimentation coefficient and the diffusion constant were calculated with the program HYDRO (Garcia de la Torre *et al.*, 1994). With this program the hydrodynamic properties of arbitrary shaped molecules can be determined from models composed of spherical beads of varying size (Garcia de la Torre, 1989, 1992; Garcia de la Torre & Bloomfield, 1981). For the work presented here we used the Fortran subroutine HYDROX, version 6 compiled on a Apple Macintosh computer. The drawings of the hydrodynamic models presented in Figure 8 were generated with the Apple Macintosh version of the ray-tracing program POV-Ray PPC 3.01.

Acknowledgments

We gratefully acknowledge the generous help and important advice of Jörg Langowski and thank Verena Weiss for valuable discussions and a gift of non-His-tagged NtrC protein for control experiments. The work presented here has been awarded the 1st European DNA award by Beckman Instruments to K.R. and was supported by DFG grant Ri 828/1.

References

Ackers, G. K., Shea, M. A. & Smith, F. R. (1983). Free energy coupling within macromolecules. The chemical work of ligand binding at the individual sites in co-operative systems. *J. Mol. Biol.* **170**, 223–242.

Austin, S. & Dixon, R. (1992). The prokaryotic enhancer binding protein NTRC has an ATPase activity which is phosphorylation dependent and DNA dependent. *EMBO J.* **11**, 2219–2228.

Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B. & Shao, Y. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science,* **277**, 1453–1474.

Bonifacio, G. F., Brown, T., Conn, G. L. & Lane, A. N. (1997). Comparison of the electrophoretic and hydrodynamic properties of DNA and RNA oligonucleotide duplexes. *Biophys. J.* **73**, 1532–1538.

Brahms, G., Brahms, S. & Magasanik, B. (1995). A sequence induced superhelical DNA segment serves as transcriptional enhancer. *J. Mol. Biol.* **246**, 35–42.

Cantor, C. R. & Schimmel, P. R. (1980). *Biophysical Chemistry. Part II: Techniques for the Study of Biological Structure and Function,* 2.3 vols, Freeman, New York.

Carey, J. (1991). Gel Retardation. *Methods Enzymol.* **208**, 103–115.

Chen, P. & Reitzer, L. J. (1995). Active contribution of two domains to cooperative DNA binding of the enhancer-binding protein nitrogen regulator I (NtrC) of *Escherichia coli*: stimulation by phosphorylation and the binding of ATP. *J. Bacteriol.* **177**, 2490–2496.

Durschlag, H. (1986). Specific volumes of biological macromolecules and some other molecules of biological interest. In *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., ed.), pp. 45–128, Springer Verlag, Berlin.

Eimer, W. & Pecora, R. (1991). Rotational and translational diffusion of short rodlike molecules in solution: oligonucleotides. *J. Chem. Phys.* **94**, 2324–2329.

Farez-Vidal, M. E., Wilson, T. J., Davidson, B. E., Howlett, G. J., Austin, S. & Dixon, R. A. (1996). Effector-induced self-association and conformational changes in the enhancer-binding protein NTRC. *Mol. Microbiol.* **22**, 779–788.

Feng, J., Atkinson, M. R., McCleary, W., Stock, J. B., Wanner, B. L. & Ninfa, A. J. (1992). Role of phosphorylated metabolic intermediates in the regulation of glutamine synthetase synthesis in *Escherichia coli*. *J. Bacteriol.* **174**, 6061–6070.

Fiedler, U. & Weiss, V. (1995). A common switch in activation of the response regulators NtrC and PhoB: phosphorylation induces dimerization of the receiver modules. *EMBO J.* **14**, 3696–3705.

Flashner, Y., Weiss, D. S., Keener, J. & Kustu, S. (1995). Constitutive forms of the enhancer-binding protein NtrC: evidence that essential oligomerization determinants lie in the central activation domain. *J. Mol. Biol.* **249**, 700–713.

Garcia de la Torre, J. (1989). Hydrodynamic properties of macromolecular assemblies. In *Dynamic Properties in Macromolecular Assemblies* (Harding, S. E. & Rowe, A. J., eds), pp. 3–31, The Royal Society of Chemistry, Cambridge.

Garcia de la Torre, J. (1992). Sedimentation coefficients of complex biological particles. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J. & Horton, J., eds), pp. 335–345, The Royal Society of Chemistry, Cambridge.

Garcia de la Torre, J. & Bloomfield, V. A. (1981). Hydrodynamic properties of complex, rigid, biological macromolecules. Theory and applications. *Quart. Rev. Biophys.* **14**, 81–139.

Garcia de la Torre, J., Navarro, S., López Martínez, M. C., Díaz, F. G. & López Cascales, J. J. (1994). HYDRO: a computer software for the prediction of hydrodynamic properties of macromolecules. *Biophys. J.* **67**, 530–531.

Gill, S. C. & von Hippel, P. H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326.

Gralla, J. D. & Collado-Vides, J. (1996). Organization and function of transcription regulatory elements. In Escherichia coli *and* Salmonella (Neidhardt, F. C., ed.), *vol. 1,* pp. 1232–1245, ASM Press, Washington, DC.

Hagerman, P. J. & Zimm, B. H. (1981). Monte Carlo approach to the analysis of the rotational diffusion of wormlike chains. *Biopolymers,* **20**, 1481–1502.

Hensley, P. (1996). Defining the structure and stability of macromolecular assemblies in solution: the re-emergence of analytical ultracentrifugation as a practical tool. *Structure,* **4**, 367–373.

Hudson, J. M. & Fried, M. G. (1990). Co-operative interactions between the catabolite gene activator protein and the *lac* repressor at the lactose promoter. *J. Mol. Biol.* **214**, 381–396.

Klenin, K., Merlitz, H. & Langowski, J. (1998). A Brownian dynamics program for the simulation of linear and circular DNA and other wormlike chain polyelectrolytes. *Biophys. J.* **74**, 780–788.

Klose, K. E., Weiss, D. S. & Kustu, S. (1993). Glutamate at the site of phosphorylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate and activates the protein. *J. Mol. Biol.* **232**, 67–78.

Klose, K. E., North, A. K., Stedman, K. M. & Kustu, S. (1994). The major dimerization determinants of the nitrogen regulatory protein NTRC from enteric bacteria lie in its carboxy-terminal domain. *J. Mol. Biol.* **241**, 233–245.

Kostrewa, D., Granzin, J., Koch, C., Choe, H. W., Raghunathan, S., Wolf, W., Labahn, J., Kahmann, R. & Saenger, W. (1991). Three-dimensional structure of the *E. coli* DNA-binding protein FIS. *Nature,* **349**, 178–180.

Kostrewa, D., Granzin, J., Stock, D., Choe, H. W., Labahn, J. & Saenger, W. (1992). Crystal structure of the factor for inversion stimulation FIS at 2.0 Å resolution. *J. Mol. Biol.* **226**, 209–226.

Kovacic, R. T. & van Holde, K. E. (1977). Sedimentation of homogenous double-strand DNA molecules. *Biochemistry,* **16**, 1490–1498.

Kuntz, I. D. & Kauzmann, W. (1974). Hydration of proteins and polypeptides. *Advan. Protein Chem.* **28**, 239–345.

Lange, R. & Hengge-Aronis, R. (1994). The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes Dev.* **8**, 1600–1612.

Laue, T. M. (1995). Sedimentation equilibrium as thermodynamic tool. *Methods Enzymol.* **259**, 427–453.

Laue, T. M., Shah, B. D., Ridgeway, T. M. & Pelltier, S. L. (1992). Computer aided interpretation of analytical sedimentation data for proteins. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J. & Horton, J. C., eds), pp. 90–125, Royal Society of Chemistry, Cambridge.

Lide, D. R. (1996). Editor of *Handbook of Chemistry and Physics,* CRC Press, Boca Raton, Fl.

Lukat, G. S., McCleary, W. R., Stock, A. M. & Stock, J. B. (1992). Phosphorylation of bacterial response regulator by low molecular weight phospho-donors. *Proc. Natl Acad. Sci. USA,* **89**, 718–722.

Magasanik, B. (1996). Regulation of nitrogen utilization. In Escherichia coli *and* Salmonella (Neidhardt, F. C., ed.), vol. 1, pp. 1344–1356, ASM Press, Washington, DC.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Merlitz, H., Rippe, K., Klenin, K. & Langowski, J. (1998). Looping dynamics of linear DNA molecules and the effect of DNA curvature: a study by Brownian dynamics simulation. *Biophys. J.* **74**, 773–779.

Mettke, I., Fiedler, U. & Weiss, V. (1995). Mechanism of activation of a response regulator: interaction of NtrC-P dimers induces ATPase activity. *J. Bacteriol.* **177**, 5056–5061.

North, A. K., Klose, K. E., Stedman, K. M. & Kustu, S. (1993). Prokaryotic enhancer-binding proteins reflect eukaryote-like modularity: the puzzle of nitrogen regulatory protein C. *J. Bacteriol.* **175**, 4267–4273.

North, A. K., Weiss, D. S., Suzuki, H., Flashner, Y. & Kustu, S. (1996). Repressor forms of the enhancer-binding protein NtrC: some fail in coupling ATP hydrolysis to open complex formation by σ^{54}-holoenzyme. *J. Mol. Biol.* **260**, 317–331.

Osuna, J., Soberon, X. & Morett, E. (1997). A proposed architecture for the central domain of the bacterial enhancer-binding proteins based on secondary structure prediction and fold recognition. *Protein Sci.* **6**, 543–555.

Pan, C. Q., Finkel, S. E., Cramton, S. E., Feng, J. A., Sigman, D. & Johnson, R. C. (1996). Variable structures of Fis-DNA complexes determined by flanking DNA-protein contacts. *J. Mol. Biol.* **264**, 675–695.

Perkins, S. J. (1986). Protein volumes and hydration effects. *Eur. J. Biochem.* **157**, 169–180.

Peters, T. (1985). Serum albumin. *Advan. Protein Chem.* **37**, 161–245.

Porschke, D. (1991). Persistence length and bending dynamics of DNA from electrooptical measurements at high salt concentration. *Biophys. Chem.* **40**, 169–179.

Porter, S. C., North, A. K., Wedel, A. B. & Kustu, S. (1993). Oligomerization of NTRC at the glnA enhancer is required for transcriptional activation. *Genes Dev.* **7**, 2258–2273.

Reitzer, L. J. & Magasanik, B. (1983). Isolation of the nitrogen assimilation regulator NRI, the product of the glnG gene of *Escherichia coli*. *Proc. Natl Acad. Sci. USA,* **80**, 5554–5558.

Reitzer, L. J. & Magasanik, B. (1986). Transcription of glnA in *E. coli* is stimulated by activator bound to sites far from the promoter. *Cell,* **45**, 785–792.

Rentzeperis, D., Rippe, K., Jovin, T. M. & Marky, L. A. (1992). Calorimetric characterisation of parallel-stranded DNA: stability, conformational flexibility, and ion binding. *J. Am. Chem. Soc.* **114**, 5926–5928.

Révet, B., Brahms, S. & Brahms, G. (1995). Binding of the transcription activator NRI (NTRC) to a supercoiled DNA segment imitates association with the natural enhancer: an electron microscopic investigation. *Proc. Natl Acad. Sci. USA,* **92**, 7535–7539.

Rippe, K., von Hippel, P. H. & Langowski, J. (1995). Action at a distance: DNA-looping and initiation of transcription. *Trends Biochem. Sci.* **20**, 500–506.

Rippe, K., Guthold, M., von Hippel, P. H. & Bustamante, C. (1997). Transcriptional activation *via* DNA-looping: visualization of intermediates in the activation pathway of *E. coli* RNA polymerase σ^{54} holoenzyme by scanning force microscopy. *J. Mol. Biol.* **270**, 125–138.

Royer, C. A. (1993). Improvements in the numerical analysis of thermodynamic data from biomolecular complexes. *Anal. Biochem.* **210**, 91–97.

Royer, C. A. & Beechem, J. M. (1992). Numerical analysis of binding data: advantages, practical aspects and implications. *Methods Enzymol.* **210**, 481–505.

Saenger, W. (1984). *Principles of Nucleic Acid Structure,* Springer-Verlag, New York.

Schuster, T. M. & Toedt, J. M. (1996). New revolutions in the evolution of analytical ultracentrifugation. *Curr. Opin. Struct. Biol.* **6**, 650–658.

Sevenich, F. W., Langowski, J., Weiss, V. & Rippe, K. (1998). DNA binding and oligomerization of NtrC studied by fluorescence anisotropy and fluorescence correlation spectroscopy. *Nucl. Acids Res.* **26**, 1373–1381.

Stafford, W. F. (1992). Boundary analysis in sedimentation transport experiments: a procedure for obtaining sedimentation coefficient distributions using the time derivative of the concentration profile. *Anal. Biochem.* **203**, 295–301.

Stafford, W. F. (1994a). Boundary analysis in sedimentation velocity experiments. *Methods Enzymol.* **240**, 478–501.

Stafford, W. F. (1994b). Sedimentation boundary analysis of interacting systems: Use of the apparent sedimentation coefficient distribution function. In *Modern Analytical Ultracentrifugation* (Schuster, T. M. & Laue, T. M., eds), pp. 119–137, Birkhäuser, Boston.

Stafford, W. F. (1997). Sedimentation velocity spins a new weave for an old fabric. *Curr. Opin. Biotech.* **8**, 14–24.

Su, W., Porter, S., Kustu, S. & Echols, H. (1990). DNA-looping and enhancer activity: association between DNA-bound NtrC activator and RNA polymerase at the bacterial glnA promoter. *Proc. Natl Acad. Sci. USA,* **87**, 5504–5508.

Tirado, M. M. & Garcia de la Torre, J. (1979). Translational friction coefficients of rigid, symmetric top molecules. Application to circular cylinders. *J. Chem. Phys.* **71**, 2581–2587.

Volkman, B. F., Nohaile, M. J., Amy, N. K., Kustu, S. & Wemmer, D. E. (1995). Three-dimensional solution structure of the N-terminal receiver domain of NTRC. *Biochemistry,* **34**, 1413–1424.

Wedel, A., Weiss, D. S., Popham, D., Dröge, P. & Kustu, S. (1990). A bacterial enhancer functions to tether a transcriptional activator near a promoter. *Science,* **248**, 486–490.

Weiss, D. S., Batut, J., Klose, K. E., Keener, J. & Kustu, S. (1991). The phosphorylated form of the enhancer-

binding protein NTRC has an ATPase activity that is essential for activation of transcription. *Cell,* **67**, 155–167.

Weiss, V. & Magasanik, B. (1988). Phosphorylation of nitrogen regulator I (NRI) of *Escherichia coli. Proc. Natl Acad. Sci. USA,* **85**, 8919–8923.

Weiss, V., Claverie-Martin, F. & Magasanik, B. (1992). Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription. *Proc. Natl Acad. Sci. USA,* **89**, 5088–5092.

Wyman, C., Rombel, I., North, A. K., Bustamante, C. & Kustu, S. (1997). Unusual oligomerization required for activity of a bacterial enchancer-binding protein. *Science,* **275**, 1658–1661.

Yuan, H. S., Finkel, S. E., Feng, J.-A., Kaczor-Grzeskowiak, M., Johnson, R. C. & Dickerson, R. E. (1991). The molecular structure of wild-type and a mutant Fis protein: relationship between mutational changes and recombinational enhancer function on DNA binding. *Proc. Natl Acad. Sci. USA,* **88**, 9558–9562.

Edited by K. Yamamoto

(Received 24 October 1997; received in revised form 9 February 1998; accepted 17 February 1998)