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Properties of a mutant form of the prokaryotic enhancer binding protein, NTRC, which hydrolyses ATP in the absence of effectors

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Abstract The mutation S170A in the proposed nucleotide binding site of the transcriptional activator protein NTRC abolishes its ability to catalyse open promoter complex formation by the σ^N-RNA polymerase holoenzyme. NTRC^{S170A} has significant ATPase activity, which, in contrast to the wild-type protein, is unaffected by phosphorylation or binding to enhancer sites on DNA. The mutant protein appears to oligomerise normally on DNA in response to phosphorylation but the ATPase activity is apparently not responsive to changes in oligomerisation state. The defect in transcriptional activation is discussed in relation to mutations in other σ^N-dependent activators.

Key words: Transcription factor; ATP hydrolysis; Phosphorylation; Enhancer binding protein

1. Introduction

The nitrogen regulatory protein NTRC is a member of the family of response regulators whose activity is controlled by phosphorylation in response to environmental signals. NTRC is an enhancer binding protein which activates transcription at promoters recognised by RNA polymerase and the alternative sigma factor, σ^N [1]. The activity of NTRC is regulated through phosphorylation of an aspartate residue (D54) in its N-terminal domain by the histidine protein kinase NTRB [2–4]. The phosphorylated form of NTRC activates transcription by catalysing the isomerisation of closed promoter complexes between σ^N-RNA polymerase and promoter DNA to open complexes in a reaction which requires ATP hydrolysis [5–7]. NTRC, in common with other σ^N-dependent activator proteins, has a highly conserved central domain containing a glycine rich motif predicted to form the phosphate binding loop of the nucleotide binding site [8,9]. This domain is required for the positive control function of NTRC and is predicted to interact with σ^N-RNA polymerase during transcriptional activation [10].

The NTRC protein has been shown to have an ATPase activity which is phosphorylation dependent and strongly stimulated by site-specific DNA binding [7,11]. Phosphorylation causes an increase in the cooperative binding of dimeric NTRC molecules to adjacent binding sites on enhancer DNA to form a tetramer or higher order oligomer. The oligomerisation of NTRC stimulates the ATPase activity of the protein which is necessary for the formation of open promoter complexes. [12,13]. Mutant forms of σ^N-dependent activator proteins with amino acid substitutions in the central domain have been described which have altered ATPase activity and fail to activate transcription [14–16]. The defect in transcriptional activation may be due to lack of ATPase activity, failure to oligomerise, inability to interact with σ^N-RNA polymerase at the promoter or failure to couple ATP hydrolysis to open complex formation. We have shown previously that a mutant NTRC protein with a single amino acid substitution, S170A, in the phosphate binding loop of the ATP binding motif cannot catalyse open complex formation to activate transcription but is apparently capable of interacting with σ^N-RNA polymerase at the *nifL* promoter [9]. We have now characterised the ATPase activity of this protein and found that it possesses the unique ability to hydrolyse ATP constitutively. The ATPase activity of the mutant protein is unusual in that it is not responsive to the oligomerisation state. The defect in transcriptional activation in this protein is therefore likely to be due to failure to couple ATP hydrolysis to open complex formation.

2. Materials and methods

2.1. Plasmid DNA and oligonucleotides

Plasmid pMS160A containing the *nifLA* promoter regulatory region has been described previously [17]. Oligonucleotides were synthesised on an Applied Biosystems 381A synthesiser and purified by polyacrylamide electrophoresis [18]. Three oligonucleotides were used to construct synthetic NTRC binding sites: A: 5′-TTGCTTTGCACTACCGCGGCCCATCCCTGCCCCAAAACGATCGCTTCAGCC-3′; B: 5′-TTGCTTTGTACTACCGCGGCCCATCCCTGCCCCAAAACGATCGCTTCAGCC-3′; C: 5′-CATCCCTGCCCCAAAACGATCGCTTCAGCC-3′.

The primer 3′-GGGTTTTGCTAGCGAAGTCGG-5′ was annealed to make the oligonucleotides double-stranded. The Klenow fragment of DNA polymerase I was used to fill in the 5′ extension in the presence of all four deoxynucleotide triphosphates. DNA templates for the gel mobility shift assays were based on the consensus enhancer binding site [12]. Two primers, 5′-GGAATTCGTTGCACTAAAATGGTGCATAATGTTAACATTAATG-3′ and 5′-CCGGATCCGTTGCACCATTTTAGTGCATTAATGTTAACATTA-3′, with complementary 3′ ends containing the strong enhancer binding sites (underlined), were annealed and extended to produce a double stranded oligonucleotide. This was cloned as an *Eco*RI-*Bam*HI fragment into pUC18 and the resultant plasmid used as a template to produce a 153bp PCR fragment using pUC18 primers 5′-ATTAAGTTGGGTAACGCCAG-3′ and 5′-AATTTCACACAGGAAACAGC-3′ complementary to the sequence on either side of the insert. The PCR fragment containing the enhancer binding sites was then itself used as a template for a further round of PCR with the same pUC18 primers to internally label the PCR product with [α-^{33}P]ATP.

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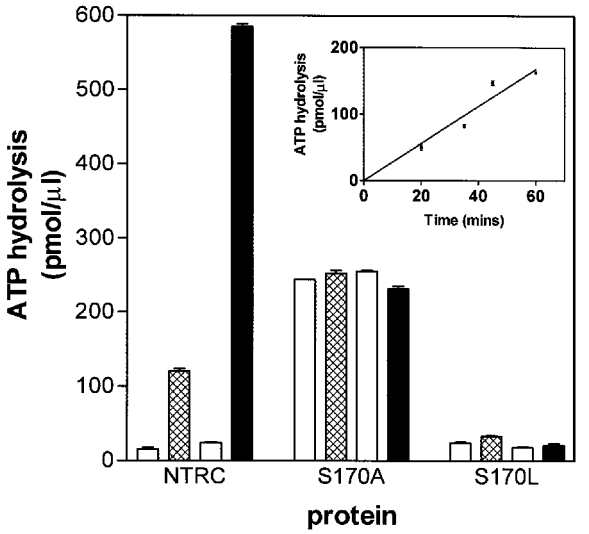

Fig. 1. Comparison of the ATPase activities of NTRC^{S170A}, NTRC^{S170L} and wild-type NTRC. Reactions containing 500 nM wild-type or mutant NTRC proteins were incubated as described in Section 2 for 1 h at 25°C in the presence or absence of pMS160A supercoiled plasmid DNA (final concentration 20 nM) and/or carbamoyl phosphate (final concentration 15 mM). Hydrolysis was initiated by addition of 3 mM [γ-^{32}P]ATP. 1 μl aliquots were removed for analysis of phosphate release by TLC as described in Section 2. Open bars indicate the absence of DNA and carbamoyl phosphate; cross-hatched bars indicate the presence of carbamoyl phosphate, grey-filled bars indicate reactions contained DNA and filled bars the presence of DNA plus carbamoyl phosphate. The inset shows a time course of ATP hydrolysis by the NTRC^{S170A} mutant protein in the absence of DNA and carbamoyl phosphate.

2.2. Proteins

Wild-type NTRC from *Klebsiella pneumoniae* was purified from extracts of *Escherichia coli* ET8894 carrying the plasmid pTH1 as described previously [19]. NTRC^{S170A} and NTRC^{S170L} were purified from overproducing strains as described previously [17] except that after MonoQ chromatography further purification was achieved by gel filtration on Superose 12 (Pharmacia). NTRB was purified as described previously [17].

2.3. ATPase assay

ATPase assays were performed in acetate buffer containing 50 mM Tris-acetate pH 7.9, 100 mM potassium acetate, 8 mM magnesium acetate, 27 mM ammonium acetate, 1 mM DTT, 3.5% PEG 6000. Final total volume was 10 μl. Phosphorylation was achieved by pre-incubation of the proteins at 37°C for 12 min with carbamoyl phosphate. ATPase reactions were initiated by addition of unlabelled ATP mixed with [γ-^{32}P]ATP (3000 Ci mmol, Amersham). Incubations were at 25°C for 1 h. The final ATP concentration in the assay was 3 mM. One tenth volume of 5% SDS, 20 mM EDTA was used to stop the reaction. The amount of ^{32}Pi released was determined by thin-layer chromatography in 0.75 M potassium dihydrogen phosphate buffer pH 3.5. 1 μl samples were spotted onto polyethyleneimine cellulose plates (Macherey-Nagel) and dried. The amount of ^{32}Pi released was quantified using a Fujix BAS1000 phosphoimager. The values obtained were expressed as pmol ATP hydrolysed per ml of reaction per hour unless stated otherwise. The concentration of NTRC was 500 nM if not otherwise stated, carbamoyl phosphate was used at a final concentration of 15 mM. Plasmid DNA was used at a concentration of 20 mM and oligonucleotides at a concentration of half the molar concentration of NTRC present in the assay.

2.4. Gel shift mobility assays

Reactions mixtures for binding assays contained 10 mM Tris acetate pH 8, 100 mM potassium acetate, 8 mM magnesium acetate, 27 mM ammonium acetate, 0.5 mM DTT, 2 mM ATP, 1.5 μg/ml poly-d(I-C) and 6 pM internally labelled probe. NTRC or NTRC^{S170A} was serially diluted in storage buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 5% glycerol, 1 mM DTT, 1 mM EDTA, 0.2 mg/ml acetylated bovine serum albumin) to give final concentrations in the assay between 0.012 and 50 nM in a final volume of 20 μl. Where present NTRB was used at 20 nM. Reaction mixtures were incubated at 37°C for 20 min, then 2 μl of loading buffer was added and the mixture loaded onto a 5% acrylamide 0.06% bisacrylamide gel running at 16 V/cm. The gel which had been pre-run for 20 min prior to loading was run in 360 mM glycine, 50 mM Tris-HCl buffer pH 8.6 for 1 h at 4°C. Gels were dried and the complexes were visualised and quantitated with a Fujix BAS1000 phosphoimager. Co-operativity factors were measured as described [20,12].

3. Results

3.1. The NTRC^{S170A} mutant protein has constitutive ATPase activity

Although NTRC^{S170A} is defective in open complex formation and cannot activate transcription, the purified protein exhibits constitutive ATPase activity. Fig. 1 shows a compar-

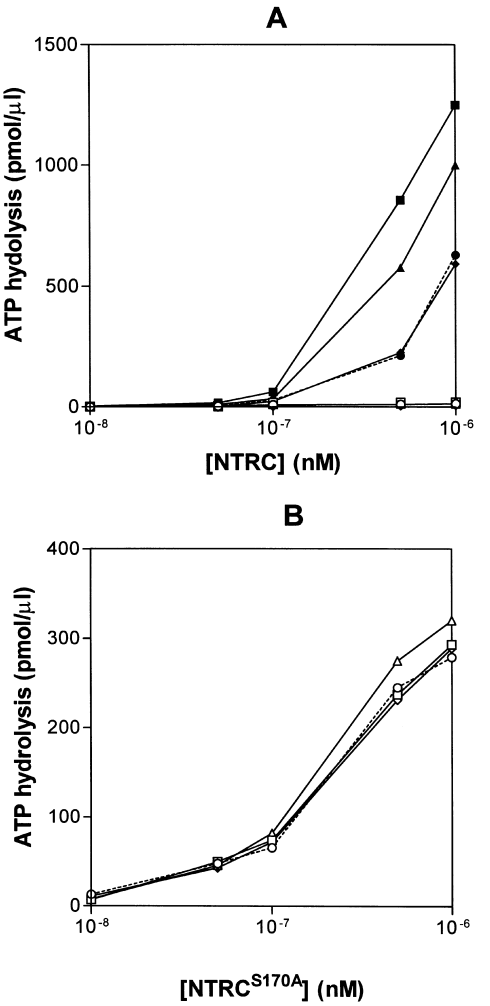

Fig. 2. Dependence of ATPase activity on NTRC protein concentration. Reactions containing the indicated amounts of wild-type NTRC (A) or NTRC^{S170A} (B) were incubated as described in Section 2 for 1 h at 25°C in the presence of double-stranded oligo nucleotides (500 nM final concentration); A (square symbols), B (triangles), or C (diamonds) or in the absence of DNA (circles, dashed line). Filled symbols indicate that carbamoyl phosphate was present where indicated at a final concentration 15 mM. 1 μl aliquots were removed for analysis of phosphate release by TLC as described in Section 2.

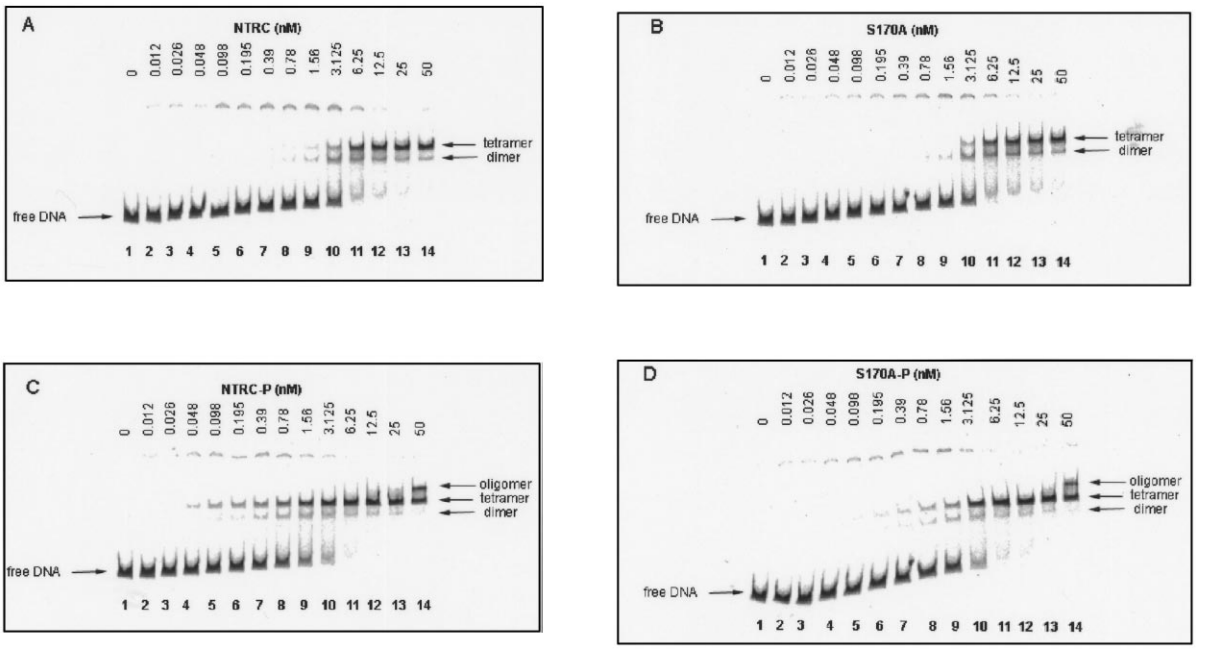

Fig. 3. Comparison of the binding of wild-type NTRC and NTRC^{S170A} to the strong *glnA* enhancer. Binding was measured using gel mobility shift assays with the 153 bp internally labelled PCR product using the pUC18 primers. The concentration of NTRC is shown above each lane. NtrB (20 nM) was present in reactions analysed in panels C and D. Co-operativity factors were determined by quantitating the relative proportions of each shifted species using a Fujix BAS1000 phosphoimager.

ison of the ATPase activities of wild-type NTRC, NTRC^{S170A} and another mutant protein with a serine to leucine substitution in the same residue, NTRC^{S170L}. As we have shown previously [11], the ATPase activity of wild-type NTRC was stimulated by phosphorylation and DNA binding, in contrast to NTRC^{S170A} which hydrolysed ATP in the absence of both effectors. Neither phosphorylation nor the presence of plasmid DNA carrying NTRC binding sites causes an increase in the level of the constitutive ATPase activity of NTRC^{S170A}. The time course of ATP hydrolysis by NTRC^{S170A} was linear for up to 1 h under the conditions of the assay and the rate was approximately 43% of that observed with the phosphorylated wild-type protein in the presence of plasmid DNA. The NTRC^{S170L} protein showed only a low background level of ATPase activity as observed with the non-phosphorylated wild-type protein under all conditions tested. S170L, like NTRC^{S170A}, is unable to activate transcription but both proteins are phosphorylated normally by NTRB [9] (and data not shown). Both NTRC^{S170A} and NTRC^{S170L} were extensively purified using the same procedure developed for the wild-type protein and showed identical chromatographic behaviour.

3.2. *The ATPase activity of NTRC^{S170A} is not influenced by the presence of the enhancer*

Wild-type NTRC is only active as an ATPase when it is able to oligomerise [12,13]. At low protein concentrations ATPase activity is observed when phosphorylation stimulates cooperative binding of dimeric NTRC molecules to adjacent sites on DNA. At higher protein concentrations higher order oligomers of phosphorylated NTRC can form in the absence of DNA and ATPase activity is observed in the absence of DNA binding. This is demonstrated in Fig. 2 which shows the effect of site-specific DNA binding on the ATPase activity of wild-type NTRC and NTRC^{S170A}. DNA binding sites were provided on short double-stranded oligonucleotides containing either the natural enhancer from the *nifL* promoter region, which contains two NTRC binding sites (sites 1 and 2, oligo A) permitting co-operative interaction between bound dimers, or alternatively a single site which is expected to bind only a dimer (site 2 only, oligo C). A third oligonucleotide (oligo B) contained the natural enhancer but with a C to T substitution in binding site 1, which has been shown previously to cause a reduction in ATPase activity compared to that observed with two wild-type sites [11]. The non-phosphorylated wild-type protein was unable to hydrolyse ATP under any of the conditions tested. The level of ATPase activity observed with the phosphorylated protein alone was similar to that in the presence of a single NTRC binding site indicating that NTRC is unable to oligomerise on this DNA template (Fig. 2A). The ATPase activity of wild-type NTRC was strongly stimulated in the presence of the oligonucleotide containing both wild-type NTRC binding sites and the stimulation was reduced in the presence of the mutant site, in accord with our previous results [11]. In contrast, the ATPase activity of NTRC^{S170A}, in either its non-phosphorylated or phosphorylated form (data not shown), was not influenced by any of the oligonucleotides (Fig. 2B). However, the response to protein concentration was slightly sigmoidal, suggestive of weak co-operative interaction in solution. Thus it would appear that oligomerisation of the NTRC^{S170A} protein is not required for ATPase activity and that unlike the wild-type protein it can probably hydrolyse ATP as a dimer. The possibility that the native NTRC^{S170A} protein already exists in an oligomeric state can be eliminated by two lines of evidence. Firstly, the molecular mass of purified NTRC^{S170A} was identical to the wild-type protein as judged by gel filtration chromatography (data not shown) and secondly, the association state of NTRC^{S170A} as measured

by sedimentation equilibrium centrifugation was similar to that of the unphosphorylated wild-type protein, both in the presence and absence of ATP (B. Davidson and T. Wilson, personal communication).

3.3. The NTRC^{S170A} mutant protein appears to assemble normally at the enhancer

The failure to observe DNA-dependent stimulation of the ATPase activity of NTRC^{S170A} could reflect either a defect in the ability of the mutant protein to interact with specific DNA binding sites or failure to bind co-operatively to more than one site to form a higher order oligomer. Previous DNAse I footprinting studies indicated that the former possibility is unlikely [9] but the cooperative binding of NTRC^{S170A} to multiple sites on DNA was not studied in detail. The ability of NTRC^{S170A} to bind cooperatively to DNA compared to wild-type NTRC was assessed by a gel mobility shift assay using PCR generated probes containing the consensus enhancer binding sites [12]. Binding of low concentrations (>50 nM) of non-phosphorylated NTRC to this enhancer gives rise to two shifted species corresponding to a single dimer and a tetramer respectively [12]; both species were observed upon binding of either wild-type or NTRC^{S170A} at similar protein concentrations (Fig. 3, compare panels A and B). The fractional maximum of the intermediate dimer bound species was also similar in both cases, corresponding to a co-operativity factor of $\sim$5 [12,20]. Quantitation of the total amount of probe shifted by all the retarded species gave similar binding curves for both non-phosphorylated proteins with half maximal binding to the DNA occurring at a protein concentration of approximately 30 nM for both NTRC^{S170A} and NTRC (Fig. 4). When phosphorylated, both proteins showed an increase in cooperative binding to the enhancer yielding more of the shifted species corresponding to the bound tetramer and the appearance of a further higher order species of unknown association state at protein concentrations of 50 nM and above (Fig. 3, compare panels C and D). Under these conditions the wild-type protein shifted the probe at lower protein concentrations than NTRC^{S170A} (Fig. 4), but quantitation of the free probe, intermediate and fully shifted species indicated a similar co-operativity factor of $\sim$45. Since the co-operativity calculations are independent of the concentration of active protein the differences in the binding isotherms possibly represent minor differences in the level or response to phosphorylation. NTRC^{S170A} therefore appears to assemble normally on DNA and phosphorylation of the mutant protein increases the cooperativity of DNA binding in a similar manner to the wild-type protein.

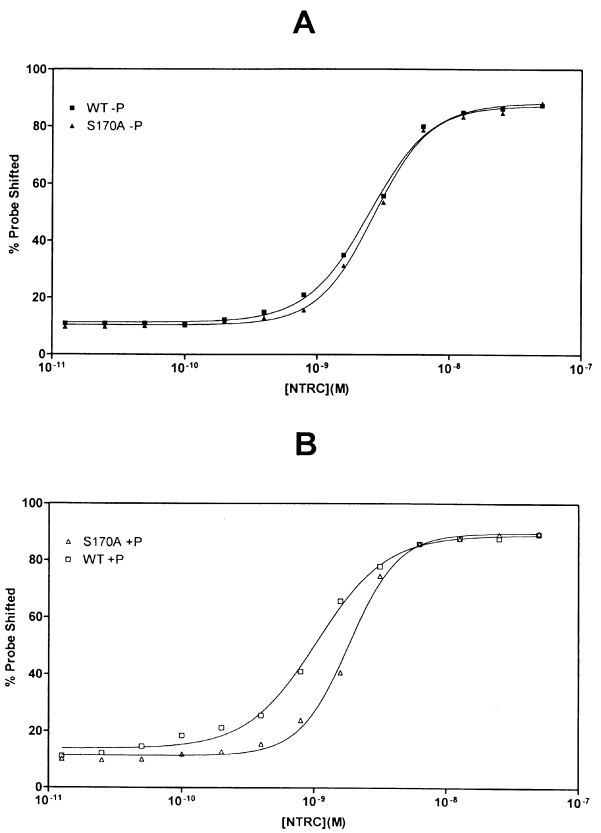

Fig. 4. Quantitation of the cooperative binding by wild-type NTRC and NTRC^{S170A} to the strong *glnA* enhancer. Data are taken from Fig. 3 and represent the total amount of shifted species as a percentage of the total radioactivity present in each lane. In panel A the binding of non-phosphorylated wild-type (closed squares) and NTRC^{S170A} proteins (closed triangles) are compared. Data in panel B are taken from Fig. 3C,D and compare wild-type (open squares) and NTRC^{S170A} proteins (open triangles) in reactions containing NTRB.

4. Discussion

The pathway which leads to the assembly of an active form of NTRC protein, capable of hydrolysing ATP is complex. Oligomerisation appears to be an essential event in the process promoted by (i) phosphorylation of the N-terminal domain which stimulates co-operative binding to multiple sites in the enhancer [11–13] and (ii) binding of ATP to the central domain which stimulates oligomerisation possibly by promoting a conformational change in the protein [15,19,21]. The dimer form of wild-type NtrC is apparently inactive as an ATPase and is not competent to activate transcription. NTRC^{S170A} appears to oligomerise under similar conditions to the wild-type protein, since phosphorylation stimulates co-operative binding to DNA and the non-phosphorylated form of the protein is not highly associated even in the presence of ATP. However the ATPase activity of the mutant protein appears to be completely uncoupled from its oligomerisation state since the unphosphorylated dimeric protein has catalytic activity, which does not apparently increase when the protein undergoes oligomerisation. The ATPase activity of the mutant protein is also uncoupled from transcriptional activation. Thus it would appear that the hydrolysis of ATP by the mutant protein is not allosterically regulated and the energy of hydrolysis cannot be utilised to drive formation of the open promoter complex by the σ^N holoenzyme.

The central activation domains of σ^N-dependent activators are well conserved and have been subdivided into seven regions of strong homology designated C1–C7 [10,22]. The region C1 is the predicted nucleotide binding site containing the glycine rich motif (GXXXXGK) found in many nucleotide binding proteins [23]. The central domain of NTRC shows homology with this motif between residues 168 and 175 and secondary structure predictions based on the EF-Tu fold as a model indicate that this region connects a β-sheet with an α-helix [9,23]. The NTRC^{S170A} mutation is not predicted to disrupt the structure of the phosphate binding loop [9]. Analo-

gous mutations (S242A and S242G) in the equivalent residue in the *K. pneumoniae* NIFA protein are also defective in transcriptional activation but the phenotype is less severe than found with $NTRC^{S170A}$ suggesting that these mutants may have significant ATPase activity [24]. However, the equivalent mutation, T175A, in the constitutively active truncated form of the *Rhizobium meliloti* DctD protein ($DctD_{(\Delta 1-142,T175A)}$) disrupts both transcriptional activation and ATP hydrolysis [25]. In the case of *K. pneumoniae* NTRC the hydroxyl side chain may be critical for regulation of ATPase activity, perhaps because it is involved in allosteric modulation via interaction with another region of the protein. Thus although the $NTRC^{S170A}$ protein can bind and hydrolyse ATP, the conformation of the protein may be altered in such a way that the dependence on oligomerisation state for ATPase activity is lost, as well as the ability to couple ATP hydrolysis to open complex formation. Although it would appear that $NTRC^{S170A}$ is able to interact with the σ^N holoenzyme, the latter defect could nevertheless be a consequence of a non-productive interaction. Substitution of the serine residue with leucine at this position (the S170L mutation) may alter the conformation of the loop so that either ATP binding or hydrolysis is impaired.

Derivatives of σ^N-dependent activators have been reported which have constitutive transcriptional activity due either to deletion of their N-terminal domains or to single amino acid substitutions in the N-terminal or central domains [26–32]. These proteins exhibit constitutive ATPase activity as a consequence of their ability to bypass the control of catalytic activity exerted by the amino-terminal domain. However, the relationship between catalytic activity and oligomerisation state has not yet been clearly defined in many of these cases. A number of other mutations in the central domains of both NTRC and other σ^N-dependent activators have been described which produce transcriptionally inactive proteins where the ATPase activities are altered or abolished by the mutation [7,14–16]. Where significant ATPase activity is observed with these mutants it appears to be coupled in the normal way to the assembly of oligomers on the appropriate enhancer binding site. In none of these cases have the mutant proteins been shown to hydrolyse ATP uncoupled to the interaction with effectors, which appears to be a unique property of the $NTRC^{S170A}$ mutation. However, some mutations in the conserved C3 region of the central domain, which is required for productive interaction with σ^N holoenzyme, have relatively high levels of ATPase activity suggesting that, like $NTRC^{S170A}$, they may fail to couple ATP hydrolysis to the formation of open promoter complexes [14,16]. Although a quantitative measure of the stoichiometry of ATP hydrolysis to open complex formation has not been reported it seems likely that $NTRC^{S170A}$ possesses enough ATPase activity to support transcription if there was no other defect. $NTRC^{S170A}$ possesses significantly more ATPase activity than the non-phosphorylated form of the $NTRC^{S160F}$ mutant protein which is fully competent to activate transcription [7,11,26]. Therefore multimerisation and ATP hydrolysis by NTRC are not the only prerequisites for activating formation of the open complex by σ^N holoenzyme, conformational changes in the activator during the catalytic cycle may be required to engage the polymerase and thus couple the energy of ATP hydrolysis to DNA strand separation.

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