Specificity and affinity of binding of phosphate-containing compounds to CheY protein

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¹H- and ³¹P-n.m.r. have been used to study the interaction of the bacterial chemotaxis protein, CheY, with ATP and a variety of other phosphates in the presence and absence of bivalent metal ions. In the metal-bound conformation, CheY will bind nucleotide phosphates and phosphates in general, while in the metal-free conformation CheY loses its affinity for phosphates. In the presence of low concentrations of nitroxide-spin-labelled ATP (SL-ATP), specific proton resonances of metal-bound CheY are suppressed, indicating that ATP binds to a specific site on this metal-bound form of the protein. These studies also show that the same resonances are affected by the binding of SL-ATP and Mn²⁺, indicating that the phosphate- and metal-binding sites are close to each other and to Asp-57 (the site of phosphorylation in CheY). ¹H- and ³¹P-n.m.r. studies using ATP, GTP, TTP, UTP, ADP, AMP and inorganic phosphates show that the binding is not specific for adenine, and does not involve the base directly, but is mediated primarily by the phosphate groups. Experiments with a phosphorylation mutant (Asp-13 → Asn) suggest that the observed phosphate binding and activation of CheY by phosphorylation may be related. Our results indicate that the conformational change and charge interactions brought about by the binding of a metal ion at the active site are required for CheY to interact with a phosphate. These studies also demonstrate the utility of spin-label-induced relaxation in conjunction with two-dimensional-n.m.r. measurements for exploring ligand-binding sites.

INTRODUCTION

Previously, it has been shown *in vitro* that the CheA, CheY, CheB and CheZ proteins involved in bacterial chemotaxis exhibit autophosphorylation and phosphate transfer properties (Hess *et al.*, 1987, 1988a,b; Oosawa *et al.*, 1988; Wylie *et al.*, 1988). In this system the CheA protein is autophosphorylated and the phosphate group is transferred to either the CheY protein or the CheB protein. The CheZ protein enhances the rate of dephosphorylation of the already unstable CheY phosphate. The signal transduction model proposed by Hess *et al.* (1987) suggests that the transfer of the phosphoryl moiety from a histidine side-chain of CheA to carboxylate side-chains of CheY activates the CheY protein, allowing it to interact with the flagellar basal body, and hence influence the sense of rotation of the motor. The CheZ protein enhances the autophosphatase activity of CheY phosphate, thereby rapidly inactivating CheY.

ATP is involved at two distinct levels in bacterial chemotaxis. First, ATP is required for the generation of *S*-adenosylmethionine (SAM), which is the methyl donor for the methylation of the transmembrane receptors during adaptation. Secondly, ATP has been shown to be required for a reaction distinct from SAM generation (Shioi *et al.*, 1982). Further, Smith *et al.* (1988) have shown the existence of a signal which is dependent on the level of the ATP pools in experiments where CheY was the only chemotaxis protein. These experiments suggest that CheY is capable of interaction productively with phosphorylated compounds other than phosphorylated CheA.

In the preceding paper (Kar *et al.*, 1992), we have shown that CheY undergoes a specific local conformational change, upon

binding of metal, close to the phosphorylation site at Asp-57. In this paper we describe ¹H- and ³¹P-n.m.r. studies used to investigate the interaction of CheY with ATP and a variety of other phosphate-containing molecules. Results from both one-dimensional and two-dimensional-n.m.r. experiments show that ATP binds close to the previously described metal-binding site, and that phosphates will bind only to the metal-bound conformation of the protein. The binding shows no specificity for adenine, and is mediated primarily by the phosphate groups rather than the base or sugar moiety. The protein also displays an interesting 'sequential' phosphatase activity [dephosphorylating ATP starting from the terminal (γ) phosphate, first producing ADP, then AMP and finally adenosine], although the observed rate under the conditions of the experiments described here is too slow to be biochemically significant. Details of experiments illustrating this activity are described in the Appendix.

MATERIALS AND METHODS

Protein purification

CheY protein was purified from an *Escherichia coli* strain carrying the plasmid pRL22 according to the method of Matsumura *et al.* (1984).

Synthesis of spin-labelled ATP (SL-ATP)

SL-ATP [3'-*O*-(1-oxyl-2,2,5,5-tetramethyl-3-carbonylpyrrolidine) ATP] was synthesized from ATP by esterification of the ribose moiety of ATP with 2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid (SL-CO₂H) (Streckenbach *et al.*, 1980). Chromatographic purification was done on a flash silica gel

Abbreviations used: CheA-P, phosphorylated CheA; SAM, *S*-adenosylmethionine; PPP, tripolyphosphate (pentasodium salt); pCH₂p, methylenediphosphonic acid; Ap[CH₂]pp, adenosine 5'-[αβ-methylene]triphosphate; App[CH₂]p, adenosine 5'-[βγ-methylene]triphosphate; SL-ATP, spin-labelled ATP [3'-*O*-(1-oxyl-2,2,5,5-tetramethyl-3-carbonylpyrrolidine) ATP]; SL-CO₂H, 2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid; bis-tris, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol; DQFCOSY, double-quantum-filtered *J*-correlated spectroscopy; HOHAHA, homonuclear Hartmann-Hahn coherence transfer spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; Me-ATP, metal ion-ATP complex; pD, pH value in ²H₂O.

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Fig. 1. Molecular structures of spin-labelled compounds: (a) the 3'-O isomer of SL-ATP; (b) SL-CO₂H

column rather than conventional silica gel, obtaining a purified yield of 39% (compared with 32% obtained by Streckenbach *et al.*, 1980). E.s.r. was used to verify that the nitroxyl free radical was present in the correct stoichiometric proportion. ¹H- and ³¹P-n.m.r. of the reduced form of SL-ATP (by the addition of sodium dithionite) were used to verify the presence of adenosine and the triphosphate respectively. ¹H n.m.r. of reduced SL-ATP indicated the presence of two isomers in the product, one being at a much higher proportion than the other. The isomers correspond to esterification at the 2' and 3' positions of the ribose. According to previous reports (Guillory & Jeng, 1977; Streckenbach *et al.*, 1980) the major product is the 3'-O isomer and this has been assumed in Fig. 1, which shows the structure of SL-ATP. Separation of the isomers was not attempted since this is not important for the results described here.

N.m.r. experiments

Purified CheY in 0.05 M-Tris buffer, pH 7.8, was concentrated by ultrafiltration at 4 °C using a YM5 disc membrane and stirred cell from Amicon (Danvers, MA, U.S.A.). The concentrated protein solution (containing ~3 mg of protein/ml) was dialysed overnight in Spectrapor membrane tubing (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) against deionized water. This removed Tris, which would otherwise give large signals in ¹H n.m.r. Water was then exchanged for ²H₂O (100.0% ²H, low in paramagnetic impurities, obtained from Aldrich, Milwaukee, WI, U.S.A.) by repeated dilution and concentration of the protein solution using the ultrafiltration cell and membrane filter. Although ²H₂O exchange is not necessary for ³¹P n.m.r., samples for both ¹H and ³¹P n.m.r. were usually treated in an identical manner so that results could be compared directly. In some of the n.m.r. experiments 0.05 M-bis-tris or Tris-d₁₁ (MSD Isotopes, St. Louis, MO, U.S.A.) in ²H₂O were used instead of ²H₂O alone, to allow better control over the pH of the sample. ATP, ADP, AMP, GTP, TTP, UTP, tripolyphosphate (PPP) and methylene diphosphonic acid (pCH₂p) were obtained from Sigma (St. Louis, MO, U.S.A.). The ATP analogues adenosine 5'-[αβ-methylene]triphosphate (Ap[CH₂]pp) and adenosine 5'-[βγ-methylene]triphosphate (App[CH₂]p) were obtained from Pharmacia (Milwaukee, WI, U.S.A.). SL-CO₂H (2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid) was obtained from Kodak (Rochester, NY, U.S.A.). These compounds were dissolved in ²H₂O, and EDTA (trisodium salt) was added to give a final concentration of 0.2–0.8 mM when added to CheY. The

pH values obtained in ²H₂O (pD) of the resultant stock solutions were adjusted to ~6.8 (±0.2) with NaO²H or ²HCl before the addition of CheY, since solubility problems arise below pD = 6.4. A pD value of < 6.8 was chosen to minimize hydrolysis of the nucleotides during the long n.m.r. experiments conducted at room temperature.

³¹P-n.m.r. spectra were recorded at 121.4 MHz on a Varian XL-300 spectrometer (Varian Associates, Palo Alto, CA, U.S.A.). Typical sample volumes were 0.7 ml in 5 mm diameter n.m.r. tubes. Spectra were recorded using the conventional one-pulse method, with a pulse recycle time of 1–3 s. The WALTZ-16 technique (Shaka *et al.*, 1983) was used for proton decoupling. Typical acquisition times were 0.5–2.5 s. The number of transients varied from 100–2000, corresponding to total acquisition times of a few minutes to ~1.5 h. All ³¹P-n.m.r. experiments were done at the ambient temperature of ~24 °C. ³¹P chemical shifts are referenced relative to the inorganic phosphate line set at 0.0 p.p.m. (+2.0 p.p.m. with respect to 85% (w/v) phosphoric acid at 24 °C, pH = 7). An exponential filtering constant of 0.2 s was used for all ³¹P-n.m.r. spectra shown here. Other experimental conditions are as described in the preceding paper (Kar *et al.*, 1992).

Measurement of dissociation constant (K_D)

K_D was calculated from the variation of ³¹P-n.m.r. line broadening as a function of substrate concentration (Dwek, 1973). In the fast-exchange limit, and for an excess of substrate, the line broadening caused by protein binding, $\delta\Delta\nu_E$, is related to the total substrate concentration, [S]₀, as follows:

$$\delta\Delta\nu_E = \frac{\Delta\nu_M[E]_0}{[S]_0 + K_D} \quad (1)$$

where $\Delta\nu_M$ is the total line width in the presence of protein-binding sites and [E]₀ is the total binding-site concentration. If [E]₀ is maintained at a constant value, then a plot of [S]₀ versus $1/\delta\Delta\nu_E$ for various [S]₀ values gives K_D as the negative intercept on the [S]₀ axis.

RESULTS AND DISCUSSION

As reported in the preceding paper (Kar *et al.*, 1992), CheY may be purified in either the metal-bound (CheY1) or the metal-free (CheY2) form. CheY2 may be converted into a metal-bound form CheY1', by the addition of a bivalent metal ion. To test whether CheY1 interacts with phosphate-containing molecules other than phosphorylated CheA (CheA-P), the effect of CheY1 on the proton resonances of adenosine and the phosphorus resonances of the phosphate groups in ATP was studied. No significant line-broadening effects were observed for the aromatic proton resonances of adenosine (spectra not shown). Nor were the transverse relaxation rates of these aromatic proton resonances affected significantly by the presence of the protein (less than 20% enhancement). However, when the same sample was examined using ³¹P n.m.r., the resonances corresponding to all three phosphate groups of ATP were found to be broadened significantly in the presence of CheY1 (Fig. 2), indicating binding of the phosphates to CheY1. These results show that the binding is mediated by the phosphates rather than by the base. The pattern of line broadening for the three phosphate groups of ATP was always $\gamma \geq \beta > \alpha$. The presence of 0.2–0.8 mM-EDTA ([EDTA] ≤ [CheY1]) did not affect the line broadening observed, indicating that it was not caused by contamination by paramagnetic metal ions. Buffer and ATP solutions were carefully treated with Chelex-100 (Bio-Rad, Richmond, CA, U.S.A.) to remove metal-ion contaminants before use.

³¹P-n.m.r. experiments with GTP, TTP, UTP, ADP and AMP

Fig. 2. ^{31}P -n.m.r. spectra showing binding of ATP to CheY

Spectra are from (a) 4.0 mM-ATP, (b) 4.0 mM-ATP + 0.8 mM-CheY1. Both samples contain 50 mM solutions of bis-tris buffer in $^2\text{H}_2\text{O}$ (pD 7.0), 0.8 mM-EDTA and 0.01% azide. Note that the line-broadening pattern is $\gamma \geq \beta > \alpha$.

Fig. 3. ^{31}P -n.m.r. spectra showing binding of PPP to CheY

Spectra are from (a) 5.0 mM-PPP; (b) 5.0 mM-PPP + 0.8 mM-CheY1. Both samples contain 50 mM-bis-tris buffer in $^2\text{H}_2\text{O}$ (pD 7.0), 0.8 mM-EDTA and 0.01% azide.

also showed line-broadening effects similar to ATP, indicating similar binding to CheY1 (spectra not shown). The aromatic protons of these nucleoside phosphates showed no significant line broadening in the presence of CheY1, confirming phosphate-mediated binding in these cases also, as with ATP. Only in the case of AMP, the ribose CH resonances showed some line broadening, and the transverse relaxation rate of the C_2H

Fig. 4. 500 MHz one-dimensional ^1H -n.m.r. spectra illustrating the specific binding of SL-ATP to CheY

Spectra are from 0.8 mM solutions of CheY1 in $^2\text{H}_2\text{O}$ (pD 6.4), containing (a) no other additives, (b) 0.20 mM-SL- CO_2H ; (c), (d) and (e) 0.05 mM, 0.10 mM, and 0.20 mM-SL-ATP respectively. Proton resonances of CheY1 that are suppressed significantly in the presence of SL-ATP are identified with arrows.

resonance of AMP was enhanced by about 50%. Structural analogues of nucleotide triphosphates, e.g. App[CH_2]p and Ap[CH_2]pp, showed similar binding properties. ^{31}P -n.m.r. experiments with PPP (Fig. 3) and p CH_2p also showed similar line-broadening effects in the presence of the protein. The transverse relaxation rates of the methylene proton resonances of p CH_2p were enhanced by about 70%, while those of the various phosphate resonances showed an enhancement of about 80–90%. Both the pattern of line broadening and the enhancement of relaxation rate results imply that it is the terminal phosphate of the triphosphates that binds to CheY1. These experiments also indicate that the base and sugar moieties are not essential for the phosphate to bind to CheY1. In fact, CheY1 will bind a single, ionized phosphate group (like sodium phosphate), with no base or sugar attached (see discussion in connection with dephosphorylation in the Appendix). At the experimental pD of about 6.8 (± 0.2) these phosphates are largely in the ionized form. Since solubility problems arise below a pD of 6.4, the binding could not be studied by n.m.r. under more acidic conditions. However, the ^{31}P resonance from a tri-substituted phosphate group, like trimethyl phosphate, remained unaffected in the presence of CheY1 (spectra not shown), confirming that charge interactions are important in the binding. No change in the chemical shifts of the ^{31}P resonances of the substrate was observed when CheY1 was added. If a phosphorylated intermediate of CheY1 is involved, it is very unlikely that it would be observable by ^{31}P n.m.r., both because of the inherent insensitivity of the detection technique, and because CheY-P is known to be highly unstable, losing the phosphate moiety in a matter of seconds by auto-dephosphorylation (Hess *et al.*, 1988b).

A strategy similar to that in the preceding paper (Kar *et al.*, 1992) was followed to explore the phosphate-binding site, using a paramagnetic nitroxide-labelled analogue of ATP (SL-ATP). Fig. 4 shows the effect of SL- CO_2H and SL-ATP on the one-

Fig. 5. Aromatic region of two-dimensional HOHAHA spectra at 30 °C (with an isotropic mixing time of 40 ms), showing the effect of SL-ATP and Mn^{2+} binding on cross-peak intensities

Spectra are from (a) 0.3 mM-CheY1' [or 0.3 mM-CheY2 + 0.5 mM- Mg^{2+} (control)]; (b) 0.3 mM-CheY1' + 0.05 mM-SL-ATP; (c) 0.3 mM-CheY1' + 0.05 mM- Mn^{2+} . All samples contain 50 mM-tris- d_{11} in

dimensional 1H n.m.r. spectra of CheY1. Qualitative comparison of spectra in Figs. (4a) and 4(b) shows that the proton resonances of CheY1 (0.8 mM in 2H_2O) are essentially unaffected by the presence of 0.2 mM-SL- CO_2H , indicating the lack of any specific binding interaction between SL- CO_2H and CheY1. However, when SL- CO_2H is covalently attached to ATP, to form SL-ATP, even 0.05 mM concentrations cause certain resonances of CheY1 to be selectively suppressed (Fig. 4c). The degree of suppression (and the enhancement of relaxation rates, as seen by measuring the transverse relaxation time, T_2) varies with the molar ratio of CheY1 to SL-ATP, as is shown in Figs. 4(c), 4(d) and 4(e), corresponding to 0.05, 0.10 and 0.20 mM-SL-ATP respectively. Control experiments with similar concentrations of ATP show no suppression of the proton resonances of CheY1, indicating that the suppression observed with SL-ATP is indeed due to the enhancement of relaxation rates of CheY1 protons close to the unpaired electron of the bound SL-ATP molecule. These results suggest that ATP binds to CheY1 at one, or a few, specific sites. The binding also appears to be significant, since even a 1:16 molar ratio of SL-ATP to CheY1 shows appreciable relaxation enhancement.

The binding specificity is illustrated more clearly in the two-dimensional HOHAHA spectra of CheY1' alone and with SL-ATP. The aromatic (Fig. 5) and aliphatic (Fig. 6) regions of the spectra are shown separately for clarity. Cross-peaks that are significantly affected by SL-ATP have been encircled. In the aromatic region (Fig. 5), cross-peaks belonging to the single tryptophan residue, Trp-58, and Tyr-106 (see Kar *et al.*, 1992 for assignments) are clearly suppressed by the presence of SL-ATP. Suppression of Trp-58 resonances implies that the phosphate must bind close to the phosphorylation site at Asp-57 at one end of the central parallel β -sheet (or the 'active-site' region) in CheY. Resonances of the four phenylalanines (8, 30, 53, 124) and Tyr-51 that form a cluster at the opposite end of this β -sheet (Volz & Matsumura, 1991) remain unaffected by SL-ATP binding. Several cross-peaks in the aliphatic region (Fig. 6) are also suppressed, in particular those belonging to Thr-87. From a consideration of spin-connectivity patterns and chemical shifts alone (Wüthrich, 1986), the other suppressed cross-peaks suggest the presence of a second threonine, at least one valine, one lysine and one or more alanines close to the phosphate-binding site. While sequential assignments of these spin systems are still incomplete, these two-dimensional experiments clearly demonstrate binding specificity, and illustrate the potential utility of spin-label-induced relaxation in two-dimensional-n.m.r. studies of binding-site geometry. Furthermore, when the suppression pattern obtained with SL-ATP binding is compared with that of Mn^{2+} binding (Figs. 5, 6 and 7), it is apparent that the phosphate- and metal-binding sites are close enough to produce similar suppression patterns. Cross-peaks of Trp-58, Thr-87 and Tyr-106 are suppressed by both SL-ATP and Mn^{2+} , although Tyr-106 appears to be closer to the nitroxide on SL-ATP than to the bound Mn^{2+} . The sharp aliphatic peak at 1.98 p.p.m. (tentatively assigned to the ϵ -methyl of a methionine), is dramatically suppressed by both SL-ATP and Mn^{2+} binding (Fig. 7), while both leave the hydrophobic cluster of residues 8, 30, 51, 53 and 124 unaffected. The second (unassigned) threonine residue, labelled THR, is suppressed to a lesser extent by SL-ATP than Mn^{2+} , while the spin system labelled X is seen to be dramatically

2H_2O , at pD 7.0. Spin connectivities for Tyr-51, Phe-53, Trp-58, Tyr-106 and Phe-124 are labelled. Cross-peaks that show significant paramagnetic suppression are encircled. Cross-peaks of Trp-58 and Tyr-106 are seen to be suppressed by both SL-ATP and Mn^{2+} , while those of Tyr-51, Phe-53 and Phe-124 are not affected by either.

Fig. 6. Aliphatic region of the two-dimensional HOHAHA spectra shown in Fig. 5

(a), (b) and (c) are as described in Fig. 5. Spin connectivity for Thr-87 is labelled. Cross-peaks of Thr-87 are seen to be suppressed by both SL-ATP and Mn^{2+} . Cross-peaks that show significant paramagnetic suppression are encircled. Note that the spin system labelled X is significantly suppressed by SL-ATP and to a smaller extent by Mn^{2+} . This spin system was shown to be affected by the T87I mutation (Fig. 5, Kar *et al.*, 1992), and its suppression by SL-ATP confirms proximity of Thr-87 to both the metal- and the phosphate-binding site. Cross-peaks of a second threonine spin system, labelled THR, [tentatively assigned to Thr-16 (Kar *et al.*, 1992)], are also seen to be suppressed by both SL-ATP and Mn^{2+} .

suppressed by SL-ATP. This spin system was shown to be affected by the T87I mutation (Kar *et al.*, 1992), and its suppression by SL-ATP binding confirms that Thr-87 is close to both the phosphate- and the metal-binding site in CheY.

It has been shown previously that the metal binding stoichiometry in CheY is 1:1 (Lukat *et al.*, 1990; Kar *et al.*, 1992). The similarity in suppression patterns observed suggests the same 1:1 stoichiometry for phosphate–CheY1 binding. Since only a single set of resonances is observed for the free and bound ligands (phosphates) in the ^{31}P -n.m.r. spectra, it may be assumed that the rate of chemical exchange between the two environments is faster than their chemical-shift difference, and that the exchange rate is in the fast-exchange region, where all the ligand nuclei resonances behave in a manner which is a weighted average of the characteristics of both environments (Dwek, 1973). Also, chemical shifts of the phosphate resonances are independent of phosphate concentration. Under these conditions the line broadening owing to protein binding, relative to the resonance line widths of the free ligand, may be expressed in terms of K_D ,

the ligand concentration and the total binding-site concentration as shown by eqn. (1). A K_D of $\sim 1.0 (\pm 0.4)$ mM is obtained for both ATP and $\text{App}[\text{CH}_2]\text{p}$ binding, using ^{31}P -n.m.r. line-broadening measurements as outlined in the Materials and methods section. The observed line-broadening effects indicate that similar K_D values would be expected for the other phosphate-containing compounds which show binding to CheY1.

Volume integrals of cross-peaks in the two-dimensional HOHAHA spectra (with and without SL-ATP) were used to estimate distances from the nitroxide radical of SL-ATP to residues close to the phosphate-binding site, as outlined in the preceding paper (Kar *et al.*, 1992). Since the e.p.r. spectra of SL-ATP in the presence of CheY1 (spectra not shown) showed that the spin label is not immobilized on binding to CheY1, the τ_r value was estimated to be about 10^{-10} s (for relevant mathematical expressions and definitions of the various terms, see Kar *et al.*, 1992). τ_c is then primarily determined by τ_r [since τ_s is about 7×10^{-6} s (Thomas *et al.*, 1976), and τ_b is about 10^{-7} s], and a value of 7×10^{-10} s is obtained for $f(\tau_c)$. The fraction of binding

Fig. 7. 500 Mhz one-dimensional ^1H -n.m.r. spectra illustrating that similar peak suppression patterns are observed for the binding of SL-ATP and Mn^{2+} to CheY

Spectra are from (a) 0.8 mM-CheY1 (control); (b) 0.8 mM-CheY1 + 0.2 mM-SL-ATP; (c) 0.5 mM-CheY1' + 0.025 mM- Mn^{2+} . All samples are in $^2\text{H}_2\text{O}$ (pD 6.4). Proton resonances that are suppressed significantly are identified with arrows.

sites, B, occupied by SL-ATP per CheY molecule, is about 0.04 under the conditions of our experiments, assuming a K_D value of 1 mM (as determined for ATP). The measured volume integrals were calibrated using Tyr-51 and Phe-124 cross-peaks, since these are not suppressed by SL-ATP. Use of appropriately scaled volume integrals of the suppressed cross-peaks gave the following estimates for distances from the nitroxide on the protein-bound SL-ATP: Tyr-106 (ring protons), 0.4–0.8 nm (4–8 Å); Tyr-106 (C_αH), 0.5–1 nm (5–10 Å); Trp-58 (C_αH), 0.5–1 nm (5–10 Å); Trp-58 (ring protons), 0.6–1.2 nm (6–12 Å); Thr(16) and X (unassigned), 0.5–1 nm (5–10 Å); Thr-87, 0.6–1.2 nm (6–12 Å). Since the nitroxide is on the ribose moiety, rather than the phosphate group, knowledge regarding the bound conformation of SL-ATP is required before these distances can be used to deduce the conformation of the phosphate-binding site. However, all of the n.m.r. relaxation rate (and line width) enhancement data are consistent with a model of SL-ATP binding to CheY 'end-on', so that it is the terminal phosphate that is tethered to the protein by its interaction with the protein-bound bivalent metal, and the adenosine is free to rotate. The nitroxide on the adenosine would then come into close proximity with several residues lying in a cone around the phosphate-binding site. The distances calculated from cross-peak suppression are consistent with this model, and the proposed metal-binding site involving Asp-57 and Asp-13 (Lukat *et al.*, 1991).

To test whether the observed non-covalent phosphate binding was related to the covalent phosphorylation of CheY, all of the experiments described above were repeated for an Asp-13 $\rightarrow$ Asn (D13N) mutant form of the protein that is known to decrease phosphorylation by more than 50% (Bourret *et al.*, 1990). Both ^{31}P -n.m.r. experiments with phosphates and ^1H -n.m.r. experiments with SL-ATP indicated binding of phosphates to the mutant protein, D13N-CheY1, but with a reduced affinity. Fig. 8 shows the effect of various concentrations of SL-ATP on the resonances of 0.4 mM-D13N-CheY1. Comparison with Fig. 4 (the equivalent figure for 0.8 mM wild-type protein) shows that more than twice the amount of SL-ATP is required to produce the same degree of suppression in D13N-CheY1 as in CheY1 (i.e. more than twice the SL-ATP/protein ratio). However, the mutation does not appear to cause any significant difference in

Fig. 8. ^1H -n.m.r. spectra illustrating the specific binding of SL-ATP to the mutant D13N-CheY1

Spectra are from 0.4 mM solutions of D13N-CheY1 in $^2\text{H}_2\text{O}$ (pD 6.4), containing (a) no other additives, (b) 0.20 mM-SL- CO_2H ; (c), (d) and (e) 0.05 mM, 0.10 mM and 0.20 mM-SL-ATP respectively. Proton resonances that are suppressed significantly in the presence of SL-ATP are identified with arrows. Comparison with Fig. 4 indicates that resonance suppression is reduced by about a factor of 2, as compared with the wild-type protein. In each spectrum, the peak at 0.04 p.p.m. is enhanced in comparison with spectra in Fig. 4, owing to the presence of a contaminant.

the conformation of the binding site: both the control spectrum and the pattern of suppression caused by SL-ATP remain essentially unchanged in one-dimensional and two-dimensional n.m.r. spectra (Figs. 8 and 9). Therefore, the reduction in resonance suppression for the mutant protein is more likely to be a reduction in binding affinity (by a factor greater than 2), rather than due to a uniform increase in distance between the bound spin label and the residues at the binding site. From consideration of charge effects alone this is a surprising result, since the neutral side-chain of Asn, containing an amide group, would be expected to provide a more favourable electrostatic environment for a negatively charged phosphate group than the negatively charged carboxylate of Asp that is replaced by Asn. However, reduced metal binding in D13N mutants of *Salmonella typhimurium* CheY indicates that Asp-13 may interact with the bound metal cation (Lukat *et al.*, 1990). Thus, replacing the negatively charged Asp-13 side-chain with a neutral Asn side-chain would disrupt charge interactions with the metal ion, and may result in the observed reduction of metal binding affinity. Our observations with D13N-CheY1 indicates that this reduced metal binding affinity correlates with a reduced binding affinity for phosphates. This explanation is consistent with the decrease in phosphorylation observed in D13N-CheY (Bourret *et al.*, 1990; Lukat *et al.*, 1990), and may indicate a possible correlation between phosphorylation and the non-covalent phosphate binding discussed here.

^{31}P -n.m.r. experiments with the metal-free form, CheY2, shows clearly that only the metal-bound form of the protein interacts with phosphates. The line widths of the three phosphate

Fig. 9. Aromatic region of two-dimensional-DQF COSY spectra at 30 °C, showing the interaction of SL-ATP with the mutant protein D13N-CheY1

Spectra are in $^2\text{H}_2\text{O}$ at pD 6.4, from 0.4 mM-D13N-CheY1 in the control spectrum in (a); and from 0.4 mM-D13N-CheY1 + 0.05 mM-SL-ATP in (b). The protein conformation and the SL-ATP binding site are seen to remain unaffected by the mutation. Cross-peaks of Trp-58 and Tyr-106 are seen to be suppressed by SL-ATP binding in the mutant protein, as with the wild-type CheY1 (see Fig. 5).

resonances of ATP remain unaffected by the presence of CheY2 (Fig. 10b). When a bivalent metal ion is added back to CheY2, producing the CheY1' species, the protein once again interacts with phosphates (Fig. 11a). The line broadening observed with CheY1' is less pronounced than that with CheY1 (Fig. 10a), indicating a lower affinity for phosphates. However, the pattern of line broadening obtained with CheY1' remains $\gamma \geq \beta > \alpha$ for the three phosphate resonances of ATP, as observed with CheY1. In these experiments with CheY1', care was taken to remove excess metal ions by ultrafiltration before the addition of ATP.

Fig. 10. ^{31}P -n.m.r. spectra at 24 °C from (a) 0.8 mM-CheY1 + 4.0 mM-ATP, and (b) 0.8 mM-CheY2 + 3.5 mM-ATP

Both samples contain 50 mM solutions of bis-tris buffer in $^2\text{H}_2\text{O}$ (pD 7.0), 0.2 mM-EDTA and 0.01% azide. Spectrum in (b) is identical to the control spectrum obtained from ATP in the absence of protein (see Fig. 2a).

All buffer solutions and the ATP stock solutions were treated with Chelex-100 to ensure removal of metal-ion impurities. The line-broadening effects observed, therefore, cannot be caused by binding of ATP to free metal ions in solution, but must be caused by binding of ATP to the metal-bound form of CheY. This is further supported by the control experiment using ATP with an equivalent amount of metal ion in the absence of protein (Fig. 11b). The pattern of line broadening observed in this case is $\beta \gg \alpha \geq \gamma$ for the three phosphate resonances, and is clearly different from that obtained with CheY1 (Fig. 10a) and CheY1' (Fig. 11a). If metal ion is added in excess to CheY1', such that the metal/protein ratio > 1 , the line-broadening pattern changes from that in Fig. 11(a) to that in Fig. 11(b), on addition of ATP, indicating the presence of a metal-ion-ATP (Me-ATP) complex.

In addition to indicating that CheY1' has a lower phosphate binding affinity than CheY1, these ^{31}P -n.m.r. experiments also demonstrate the lower metal binding affinity of CheY1' (Kar *et al.*, 1992). On addition of excess ATP (or phosphates), such that the phosphate/CheY1' ratio is > 1 , the line-broadening pattern changes from that observed in Fig. 11(a) to that in Fig. 11(b), and the CheY ^1H spectra show an increase in CheY2 content, indicating that excess ATP will extract the metal ions from the protein. However, for CheY1, the phosphate line-broadening pattern remains unchanged even at a 10:1 ratio of phosphate/CheY1, i.e. the metal ion remains bound to the protein even in the presence of excess phosphates. ^1H n.m.r. also indicates that CheY1 remains in the metal-bound conformation in the presence of excess phosphates. A K_D value of 0.045 ± 0.008 mM has been reported for the Mg-ATP complex at 25 °C and pH = 7.2 (Gupta *et al.*, 1978). Since the protein-bound Mg^{2+} is not extracted from CheY1, even in the presence of excess ATP, the K_D of Mg-CheY1 must be lower than 0.045 mM, or more than an order of magnitude lower than that measured for Mg-CheY1' ($K_D \approx 0.6$ mM). Thus, once EDTA has been used to obtain the metal-free form of the protein, binding of any subsequently

Fig. 11. ^{31}P -n.m.r. spectra at 24°C from (a) $0.8\text{ mM-CheY2} + 0.8\text{ mM-Mg}^{2+} + 0.8\text{ mM-ATP}$, and (b) $10\text{ mM-Mg}^{2+} + 10\text{ mM-ATP}$

Both samples contain 50 mM solutions of bis-tris buffer in $^2\text{H}_2\text{O}$ (pD 7.0), 0.2 mM-EDTA and 0.01% azide. Note that the line-broadening pattern for the three phosphate resonances in (a) is $\gamma \geq \beta > \alpha$, while in (b) it is $\beta \gg \alpha \geq \gamma$.

added metal is somewhat weakened in comparison with the native form. This decrease in the metal binding affinity of CheY1' correlates with its decreased affinity for phosphates, as observed with the D13N mutant of CheY. While a change in charge interactions at the metal-binding site of D13N-CheY1 may explain the observed decrease in its metal (and consequently phosphate) binding affinity, a similar explanation is not applicable for the decrease observed in CheY1'. A subtle difference in conformation at the metal-binding site appears to be responsible in the case of CheY1', judging from the small differences in ^1H -n.m.r. chemical shifts of nearby Trp-58 and Tyr-106 residues (Kar *et al.*, 1992). However, the nature of this conformational difference between CheY1 and CheY1' is not clear. Since CheY1', obtained from freshly prepared CheY2 by the addition of bivalent metal, shows phosphate-binding properties that are distinctly different from those of freshly prepared CheY1, irreversible degradation of the protein does not appear to be the cause of these observed differences. Also, CheY1, CheY2 and CheY1' are all stable at low temperatures (0 – 10°C) for extended periods (over 1 month).

The results described here show that the interaction of CheY with phosphates provides an interesting model for the interaction of CheY with CheA-P. Although no phosphorylated intermediate of CheY is observed, these experiments clearly show that the bound metal is critical for maintaining the appropriate charge environment and conformation for the interaction of phosphates with CheY.

In summary, a spin-labelled analogue of ATP has been used to show that ATP binds to the chemotaxis protein CheY at a location close to the metal-binding site. A combination of ^1H - and ^{31}P -n.m.r. experiments with a variety of phosphates suggest that the binding is mediated primarily by the phosphate groups. Charge interactions are observed to be particularly important for

phosphate binding. From the dependence of n.m.r. line width on substrate concentrations, K_D for this binding is calculated to be $\sim 1\text{ mM}$. Interaction with phosphates requires the protein to be in the metal-bound conformation. The phosphorylation mutant D13N shows reduced affinity for phosphates, but the mutation does not seem to affect the conformation of the protein significantly. Two-dimensional n.m.r., in conjunction with paramagnetically enhanced relaxation experiments, shows clearly that both metal and phosphate bind close to the phosphorylation site at Asp-57 in CheY, and that Thr-87, Tyr-106 and a methionine are also close to this active site.

Our results demonstrate that, when combined with two-dimensional n.m.r., paramagnetically induced relaxation is a potentially powerful technique for the investigation of ligand-binding interactions, and may provide useful information regarding the binding site, even without complete structural elucidation by n.m.r. Spin-labelled ligands and paramagnetic metals have been used extensively in one-dimensional n.m.r. work, but appear to have been used in only a few exploratory two-dimensional-n.m.r. studies (Kosen *et al.*, 1986; de Jong *et al.*, 1988; Kosen, 1989). The spectra shown in Figs. 5 and 6 exhibit very selective suppression of only a few cross-peaks. Distances estimated from the relative suppression of cross-peak intensities in the presence of SL-ATP (and Mn^{2+}) are consistent with the current model of metal binding at the active site. More complete sequential assignments for all suppressed resonances should lead to the development of a detailed model of binding-site geometry. The aim now must be to develop such a model for binding of phosphates and metal to CheY.

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APPENDIX

Our investigation of CheY-P interactions by ^{31}P n.m.r. also led to the discovery of a slow phosphatase activity in CheY1. In addition to the line-broadening effects discussed above, extra resonances were observed in the ^{31}P -n.m.r. spectra of ATP after incubation with CheY1 (Fig. A1). Two new resonances (at 0 and -12 p.p.m.) appear initially, after several hours at room temperature. These were assigned to inorganic phosphate (P_i) and the α -P of ADP (Fig. A1b). The intensities of both resonances were observed to increase with time. The control sample (ATP in $^2\text{H}_2\text{O}$) showed no evidence of degradation over the same period of time, and was observed to be stable at 4°C for several weeks. To check whether dephosphorylation would continue beyond the loss of the γ -P of ATP, the sample was stored at 4°C and examined by ^{31}P n.m.r. after 3 days. Intensities of resonances

associated with P_i and ADP were seen to increase, and a new resonance at ~ 2 p.p.m. was observed, indicating the presence of AMP (Fig. A1c). An independent experiment with AMP showed that CheY1 binds to and dephosphorylates AMP also, producing P_i (spectra not shown). CheY1 is thus able to dephosphorylate ATP completely, producing adenosine. As with phosphate binding, this dephosphorylation is not base specific, and any phosphate that shows binding to CheY1 is also dephosphorylated. For instance, PPP is dephosphorylated to pyrophosphate (PP_i) and P_i (Fig. A2). The ATP analogues App[CH_2]p and Ap[CH_2]pp show line-broadening effects similar to those of ATP, indicating binding of these structural analogues to CheY1 (Figs. A3 and A4). After several hours of incubation with CheY1 at 24°C , dephosphorylation was observed in the case of Ap[CH_2]pp but not App[CH_2]p, indicating that CheY1 removes only the terminal phosphate. The small peak at ~ 0 p.p.m. in Fig. A3(a)

Fig. A1. ^{31}P -n.m.r. spectra showing binding and subsequent dephosphorylation of ATP

Spectra are from 50 mM solutions of bis-tris buffer in $^2\text{H}_2\text{O}$ (pD 7.0), containing (a) 5 mM-ATP; (b) 4 mM-ATP+0.5 mM-CheY1; (c) 5 mM-ATP+0.8 mM-CheY1. Spectrum (b) was recorded after incubation for ~ 9 h at room temperature. Spectra in (a) and (c) were recorded after an additional incubation of 3 days at 4°C ; the spectrum in (a) remained unchanged over this time period.

Fig. A2. ^{31}P -n.m.r. spectra showing binding and dephosphorylation of PPP

Spectra are from 5.0 mM solutions of PPP in 50 mM-bis-tris buffer in $^2\text{H}_2\text{O}$ (pD 7.0), containing (a) no other additives, or (b) and (c) 0.8 mM-CheY1. Spectrum (b) was recorded after incubation for ~ 0.5 h at room temperature. Spectra (a) and (c) were recorded after 3 days of incubation at 4°C .

Fig. A3. ^{31}P -n.m.r. spectra showing the inability of CheY1 to dephosphorylate the ATP analogue, $\text{App}[\text{CH}_2]\text{p}$

Spectra are from 3 mM solutions of $\text{App}[\text{CH}_2]\text{p}$ in 50 mM-bis-tris buffer in $^2\text{H}_2\text{O}$ (pD 7.0), containing (a) no other additives, or (b) and (c) 0.5 mM-CheY1. Spectrum (b) was recorded after incubation for ~12 h at room temperature. Spectra (a) and (c) were recorded after an additional incubation of 3 days at 4 °C. Line broadening in (b) and (c) show that the CheY1 does bind $\text{App}[\text{CH}_2]\text{p}$, but the substrate is not dephosphorylated. The small sharp peak at ~0 p.p.m. is due to P_i contamination present in the commercial material at the beginning of the experiment, and its intensity does not increase with time.

is due to P_i contamination present in the commercial materials; this peak is not observed to grow with time. Possible products from the random dephosphorylation (at the α , β or γ position) of $\text{App}[\text{CH}_2]\text{p}$ are P_i , $\text{pp}[\text{CH}_2]\text{p}$, AMP and pCH_2p . None of these products is observed in the ^{31}P -n.m.r. spectrum even after several days at 4 °C (Fig. A3). Dephosphorylation of $\text{Ap}[\text{CH}_2]\text{pp}$ by CheY1 produces only $\text{Ap}[\text{CH}_2]\text{p}$ and P_i (Fig. A4). Several days of incubation at 4 °C only results in increased relative intensities of resonances associated with $\text{Ap}[\text{CH}_2]\text{p}$ and P_i . No other products are observed, indicating that $\text{Ap}[\text{CH}_2]\text{pp}$ (and $\text{Ap}[\text{CH}_2]\text{p}$) cannot be dephosphorylated at the α -position to give adenosine and $\text{p}[\text{CH}_2]\text{pp}$ (and pCH_2p). If the terminal phosphate does not involve an ester linkage, it cannot be removed by CheY1 and dephosphorylation of the substrate is arrested at that stage. The resonance lines corresponding to the products of dephosphorylation also showed line broadening (in comparison with controls in the absence of CheY), indicating that NDP, NMP, PP_i and P_i bind to CheY1, and that CheY1 will bind a single, ionized phosphate group, with no base or sugar attached. For example, the peak at ~0 p.p.m. in Fig. A3, caused by P_i contamination, shows line broadening in the presence of CheY1, indicating that P_i binds to CheY1.

The rate of substrate dephosphorylation was found to be very

Fig. A4. ^{31}P -n.m.r. spectra showing the binding and dephosphorylation of the ATP analogue $\text{Ap}[\text{CH}_2]\text{pp}$

Spectra are from 3 mM solutions of $\text{Ap}[\text{CH}_2]\text{pp}$ in 50 mM-bis-tris buffer in $^2\text{H}_2\text{O}$ (pD 7.0), containing (a) no other additives, or (b) and (c) 0.5 mM-CheY1. The spectrum (b) was recorded after incubation for ~12 h at room temperature. Spectra (a) and (c) were recorded after an additional incubation of 3 days at 4 °C.

slow under the conditions of the experiments described here (~10 nM s^{-1} at 0.5 mM-CheY1 and 4 mM-ATP, corresponding to a k_{cat} of ~10⁻⁵ s^{-1}). Conditions of pH, ionic strength, etc., that may affect this rate have not been fully explored and it is not known whether a biochemically meaningful rate may be obtained under optimum conditions. Experiments with alkaline phosphatase (Coleman, 1987) have shown that the rate of dephosphorylation may be slowed down by several orders of magnitude under experimental conditions different from those required *in vivo*. That the substrate dephosphorylation is not caused by a contaminant has been ensured by verifying that this activity tracks the CheY1 profile. Also, when the protein is isolated in the metal-free form as CheY2 (or the bound metal is removed from CheY1 by excess EDTA), no phosphatase activity is observed, showing that both phosphate binding and dephosphorylation require the metal-bound form of the protein.

The CheY homologue, NtrC, has been shown to exhibit an ATPase activity which is required for protein function and is regulated several-hundred-fold by the phosphorylation state of the protein (Weiss *et al.*, 1991). The ATPase activity in NtrC is thought to be associated with the nucleotide-binding region in the central activation domain, rather than the *N*-terminal regulatory domain that is homologous to CheY. However, it is possible that the observed low level of phosphatase activity of CheY may be stimulated in the phosphorylated state in a manner analogous to the ATPase activity of NtrC. Unfortunately, CheY-P is highly unstable and the phosphatase activity of the phosphorylated form would be difficult to measure.

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