Bivalent-metal binding to CheY protein

Effect on protein conformation

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CheY is a 14 kDa cytoplasmic protein that is activated by the transfer of a phosphoryl moiety to Asp-57 from phosphoCheA during signal transduction in bacterial chemotaxis. It has been established that metal ions are necessary for the autophosphorylation of CheA, the transfer of phosphate from phosphoCheA to CheY and the autodephosphorylation of phosphoCheY. In this work, paramagnetic relaxation enhancement has been used in conjunction with one- and two-dimensional n.m.r. to study the interaction of CheY with bivalent metal ions. These studies have led to the discovery of two conformations of the protein in water, corresponding to the metal-free and the metal-bound states. Binding of bivalent cations like Mg^{2+} , Ca^{2+} , Sr^{2+} , Zn^{2+} and Mn^{2+} results in a conformational change from the metal-free to the metal-bound state. Preliminary assignments of the aromatic proton resonances are reported. Comparison of phase-sensitive double-quantum-filtered COSY, homonuclear Hartmann–Hahn coherence transfer and nuclear Overhauser enhancement spectra from the metal-bound and metal-free protein indicates that Trp-58, Thr-87 and Tyr-106 are particularly affected by the conformational change involved, and that this change is limited to a small number of residues. In addition, homonuclear Hartmann–Hahn coherence transfer experiments with paramagnetic Mn^{2+} show significant suppression of cross-peaks associated with Trp-58 and several neighbouring residues. Comparison of the distances estimated using n.m.r. with the CheY crystal structure indicates that the n.m.r. results are consistent with bivalent metal binding at the cluster of aspartic acid residues that includes Asp-13 and Asp-57. These studies also demonstrate the utility of paramagnetic metal-induced relaxation in conjunction with two-dimensional n.m.r. measurements for exploring ligand-binding sites.

INTRODUCTION

Bacteria are able to sense and respond to changes in their environment by swimming away from unfavourable conditions or toward attractants (Stewart & Dahlquist, 1987; Bourret *et al.*, 1991; Stock *et al.*, 1991). Such chemotactic behaviour is usually mediated by a series of transmembrane receptor proteins that monitor environmental conditions by interacting with specific chemicals. Changes in the environment detected by these receptors are transduced into two kinds of intracellular signals: excitation and adaptation. The excitation signal regulates the direction of rotation of the bacterial flagella, altering the ‘tumbling’ or ‘smooth swimming’ behaviour of the cell. The adaptation signal acts as a feedback mechanism to modulate the behaviour of the transmembrane receptors by specific structural modifications. The genes and gene products responsible for chemotaxis and signal transduction in bacteria have been identified and characterized over the past decade. Based on this work, the following model for chemotaxis has been suggested. Changes in the environment cause a transmembrane receptor protein to signal the cytoplasmic protein, CheA, which responds by altering its rate of autophosphorylation. Increase in the concentration of phosphorylated CheA (CheA-P) leads to the rapid transfer of phosphate to CheY. Phosphorylated CheY (CheY-P) interacts with a ‘switch’ at the base of the flagellum and generates a reversal of flagellar rotation from counterclockwise to clockwise. Another cytoplasmic protein, CheZ, enhances the rate of auto-

dephosphorylation of CheY-P, thus regulating its concentration and inactivating the flagellar switch. CheA-P also phosphorylates CheB, yet another cytoplasmic component of the chemotaxis system. CheB acts as a feedback system to modulate the activity of the transmembrane receptor protein via a reversible demethylation/methylation process.

CheY-P, therefore, is the signal that regulates the direction of the flagellar motors. As such, CheY forms the excitation part of the transduced signal, and its phosphorylation/dephosphorylation plays a central role in signal transduction. It is of prime importance to determine how phosphorylation activates CheY, and subsequently why CheY-P but not CheY causes a clockwise rotation of the flagellar motor. It is known that CheA is autophosphorylated at a histidine residue in the presence of ATP and excess Mg^{2+} (Hess *et al.*, 1988a), and that CheA-P phosphorylates CheY at Asp-57 (Sanders *et al.*, 1989). It has been shown that EDTA stops the phosphotransfer from CheA-P to CheY (Hess *et al.*, 1988b). The phosphorylation assay used by Hess *et al.* requires excess Mg^{2+} , and although the metal-ion specificity was not clearly established, Hess *et al.* (1988b) indicated that Mg^{2+} is required for the autodephosphorylation of CheY-P in the absence of CheZ. It is therefore likely that metal ions play an important part in both the phosphorylation and the dephosphorylation processes.

More recently, Lukat *et al.* (1990) have used fluorescence spectroscopy to show that CheY contains a bivalent-metal-ion binding site. Reduced metal binding to the D13N mutant (Asp-

Abbreviations used: CheA-P, phosphorylated CheA; CheY-P, phosphorylated CheY; CheY1, native metal-bound CheY protein; CheY2, metal-free CheY; CheY1', metal-bound CheY obtained by addition of bivalent metal ions to CheY2; *Ec*-CheY, CheY from *Escherichia coli*; *St*-CheY, CheY from *Salmonella typhimurium*; bis-tris, 2,2-bis(hydroxymethyl)-2,2',2'-nitrilotriethanol; DSS, 4,4'-dimethyl-4-silapentane-1-sulphonate; DQFCOSY, double-quantum-filtered *J*-correlated spectroscopy; HOHAHA, homonuclear Hartmann–Hahn coherence transfer spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; pD, pH values with ^2H_2O ; AA, atomic absorption spectroscopy; NOE, nuclear Overhauser enhancement.

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13 replaced by Asn) and lack of metal binding to the D57N mutant (Asp-57 replaced by Asn) indicated that the acidic pocket formed by Asp-13 and Asp-57 (A. M. Stock *et al.*, 1989) is involved in metal binding. It was also shown that the metal ion is required for phosphotransfer from CheA-P to CheY and for the dephosphorylation of CheY-P. The work described here uses one-dimensional and two-dimensional n.m.r. studies to demonstrate that CheY exhibits two conformations corresponding to the metal-free and metal-bound forms, and shows direct evidence of metal binding close to the phosphorylation site at Asp-57. In addition, it provides the first set of ^1H -n.m.r. resonance assignments for this 14 kDa chemotaxis protein.

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).

N.m.r. experiments

The purified fraction of CheY in 0.05 M-Tris buffer at 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 $^2\text{H}_2\text{O}$ (100.0% ^2H , low in paramagnetic impurities, from Aldrich, Milwaukee, WI, U.S.A.) by repeated dilution and concentration of the protein solution using the ultrafiltration cell and membrane filter. The pH values of $^2\text{H}_2\text{O}$ (pD) were adjusted with NaOH or ^2HCl before dialysis ($^2\text{H}_2\text{O}$ exchange). The pD values reported here are direct readings from the pH meter, with no correction for the effect of the deuterium isotope on the electrode. Protein concentration was measured optically, using an IBM 9420 u.v./visible spectrophotometer to monitor the absorbance at 280 nm. The absorbance was first calibrated with known CheY concentrations, determined by the standard Bradford protein dye-binding assay (Bradford, 1976), using the Pierce protein assay kit (Rockford, IL, U.S.A.) and BSA as the reference protein (Sigma, St. Louis, MO, U.S.A.). Initially, 0.01% azide was included in all n.m.r. samples to prevent bacterial growth. However, it was found that maintaining the protein in $^2\text{H}_2\text{O}$ is itself a very effective deterrent for bacterial growth, and azide was not always included in $^2\text{H}_2\text{O}$ -exchanged samples.

Metal-free protein samples were prepared by washing the native protein several times with 10 mM-EDTA in H_2O (or $^2\text{H}_2\text{O}$) in a stirred cell over a YM5 disc membrane. The EDTA was then removed either by dialysis or by repeated washings with the required solvent ($\text{H}_2\text{O}/^2\text{H}_2\text{O}$) in a similar fashion. Only paramagnetically pure $^2\text{H}_2\text{O}$ was used, to avoid metal-ion impurities. Stock solutions of azide, buffers, etc., in $^2\text{H}_2\text{O}$, were first treated with Chelex-100 (Bio-Rad, Richmond, CA, U.S.A.) to remove metal-ion contaminants.

Conformational changes on binding of metal ions were checked by n.m.r. by the addition of appropriate salt solutions in $^2\text{H}_2\text{O}$, after adjusting the pD values to that of the protein solution. Chlorides of calcium, magnesium, strontium, zinc and manganese were used. Generally, azide was not included in these samples, since calcium precipitates out as calcium azide. Chelex-100-treated deuterated Tris buffer (Tris- d_{11} , MSD Isotopes, St. Louis, MO, U.S.A.) was usually used for these experiments (50 mM in $^2\text{H}_2\text{O}$, pD = 7.0 ± 0.1) to maintain a stable pD value.

The one-dimensional and two-dimensional ^1H -n.m.r. spectra at 500 MHz were recorded on General Electric Ω 500 and GN500 spectrometers. Typical sample volumes were 0.5 ml in 5 mm diameter n.m.r. tubes (528-PP from Wilmad, Buena, NJ, U.S.A.) One-dimensional ^1H -n.m.r. spectra were recorded using either conventional one-pulse, or one-pulse with solvent presaturation, techniques. In the latter, the decoupler, set at the frequency of the residual water resonance, was turned on during the pulse recycle time of 3–4 s. Typical acquisition times were 2.7 s. Each spectrum represents a time average of 16 scans. The one-dimensional spectra presented here were recorded at approx. 20 °C. Chemical shifts are referenced to the residual water signal [4.82 p.p.m. relative to 4,4'-dimethyl-4-silapentane-1-sulphonate (DSS) at 20 °C]. An exponential filtering constant of 0.5 s was used for all one-dimensional ^1H -n.m.r. spectra.

Two-dimensional double-quantum-filtered *J*-correlated spectroscopy (DQFCOSY) (Rance *et al.*, 1984), two-dimensional homonuclear Hartmann–Hahn coherence transfer spectroscopy (HOHAHA) (Braunschweiler & Ernst, 1983; Bax & Davis, 1985) and nuclear Overhauser enhancement spectroscopy (NOESY) (Jeener *et al.*, 1979; Macura *et al.*, 1981) experiments were performed in the phase-sensitive hypercomplex mode (States *et al.*, 1982). Presaturation during the preparation period of 1.2–2.0 s was used to suppress the solvent peak. In the NOESY experiments, the decoupler was also turned on during the mixing time. The HOHAHA experiments used a MLEV17 synchronous decoupler modulation of 12.5 kHz for spin-lock and no trim pulses. Typically, a sweep width of 6250 Hz was used, with 1024 complex data points and 256–512 t_1 increments. The spectra were processed to 1024×1024 real points with FTNMR (or FELIX) software from Hare Research Inc. (Woodinville, WA, U.S.A.) on a microvax or Silicon Graphics workstation. NOESY and DQFCOSY spectra were resolution-enhanced in t_1 and t_2 by phase-shifted sine-bell or sine-bell-square apodization windows. A skewed sine-bell was used for HOHAHA spectra. Baseline correction was performed in both dimensions. The two-dimensional spectra presented here were recorded at 30 °C. Chemical shifts are referenced to the residual water signal (4.72 p.p.m. relative to DSS at 30 °C).

Fluorescence experiments

Steady-state fluorescence experiments were done on a Spex Fluorolog 2 spectrofluorimeter (SPEX Inds. Inc., Edison, NJ, U.S.A.) (with a xenon arc lamp, and a double-grating spectrometer in the excitation position), operating under the control of a Spex Datamate DM1B multiprocessor. An excitation wavelength of 295 nm was chosen for selective excitation of the single tryptophan residue, Trp-58, in CheY from *E. coli* (Ec-CheY), the other fluorescent residues being Tyr-51 and Tyr-106. Absorbance at the excitation wavelength was less than 0.1 for all samples. L-Trp in 4 mM-sodium phosphate buffer at pH 5.5 with a quantum yield of 0.147 (Wiget & Luisi, 1978) was used as reference for the estimation of quantum yield values for the wild-type and the T87I mutant CheY protein. The dissociation constant (K_D) for magnesium binding was obtained from tryptophan fluorescence experiments with added magnesium. Since the added metal did not alter the fluorescence profile, only the change in fluorescence intensity at the emission peak at 340 nm was monitored at various metal ion concentrations. The binding stoichiometry, n , and K_D were calculated from a linear least-squares fit to the Scatchard equation for equilibrium binding (Fersht, 1985):

$$\nu = n - K_D \frac{\nu}{[L]},$$

where ν is the number of mol of ligand bound per mol of protein,

n is the number of identical non-interacting binding sites per protein molecule and $[L]_f$ is the concentration of free ligand. The quenching experiments were done at 20 °C, using 5–10 μ M protein solution in 50 mM-Tris at pH 7. Several stock solutions of magnesium were used to scan a large range of metal ion concentrations (micro- to milli-molar) without significant dilution of the protein solution. Results were corrected for any dilution.

Estimation of distances from peak suppression

The fractional amplitude, A_{tr} , of a singlet proton resonance line of width $\Delta\nu_c$ in the absence of any paramagnetic species is given by

$$A_{tr} = \frac{\Delta\nu_p}{\Delta\nu_c + \Delta\nu_p} \quad (1)$$

where $\Delta\nu_p$ is the paramagnetic contribution to the line width in the presence of a paramagnetic probe. The distance between the paramagnetic centre and the proton may be expressed in terms of the paramagnetic contribution to the relaxation rate, $(T_{2p})^{-1}$ (Krugh, 1976):

$$r(\text{\AA}) = C[f(\tau_c)T_{2p}]^{\frac{1}{3}} \quad (2)$$

where $f(\tau_c)$ is a function of the rotational correlation time, τ_c , of the vector between the paramagnetic centre and the proton in question, and the proton and electron Larmor frequencies. The constant, C , contains the dipolar interaction, and has a value of 480 for protons, when r is in $\text{\AA}$ ngstroms ($1 \text{\AA} = 0.1 \text{ nm}$) and T_{2p} is in seconds. Noting that $(T_2)^{-1} = \pi\Delta\nu$, the distance may be expressed in terms of $\Delta\nu_p$:

$$r(\text{\AA}) = C \left[f(\tau_c) \frac{B}{\pi\Delta\nu_p} \right]^{\frac{1}{3}} \quad (3)$$

where B is the fraction of binding sites occupied by the paramagnetic species per protein molecule. B may be calculated from the protein and paramagnetic ligand concentrations and the dissociation constant, using the mass action expression. The sixth-root dependence in eqn. (3) allows fairly precise determination of r , so that even a factor of 10 uncertainty in the product $[f(\tau_c)B/\pi\Delta\nu_p]$ produces only about a factor of two uncertainty in r .

RESULTS AND DISCUSSION

Native CheY, purified from *E. coli* by the method of Matsumura *et al.* (1984), may be isolated in either the metal-bound (CheY1) or the metal-free (CheY2) form, and show distinctly different proton n.m.r. spectra (compare Figs. 1b and 1a), indicating different protein conformations. Two regions of significant differences between the spectra from CheY1 and CheY2 are around 7.5–7.2 p.p.m. and 5.9–5.4 p.p.m. Addition of a bivalent metal ion, such as magnesium, converts CheY2 into a metal-bound form (CheY1'). The one-dimensional proton n.m.r. spectrum of CheY1' is essentially identical to that of CheY1 (compare Figs. 1b and 1c), indicating the presence of bound metal in the native form of CheY. Addition of calcium, strontium or zinc gives spectra identical to the magnesium-bound form, showing that the observed conformational change is not specific to magnesium ions. While CheY2 may always be obtained from CheY1 by the removal of the bound metal ions with excess EDTA, the native form cannot be regained completely by the addition of any single type of metal ion back to CheY2. Differences are noticeable between the spectra of the two metal-bound forms, CheY1 and CheY1', particularly at p.p.m. values of 5.90, 5.50 and 1.98 (Figs. 1b and 1c). These differences are

more noticeable in the two-dimensional spectra, which are almost superimposable, except for differences in chemical shifts of a small number of cross-peaks.

The primary sequence for this 14 kDa, 128-residue protein has been published (Matsumura *et al.*, 1984). The nine aromatic residues in CheY consist of one tryptophan, two tyrosines and six phenylalanines. Cross-peak assignment has been completed for all the ring protons of these nine aromatic residues. For some of these residues, the C_{α} , C_{β} and amide protons have also been identified and sequential assignment has been completed. A list of assigned resonances is shown in Table 1 and the details of the assignment procedure are given below.

Resonance assignments

The two tyrosines were easily recognized by the single cross-peak between the *ortho* and *meta* proton resonances in HOHAHA spectra in $^2\text{H}_2\text{O}$ at 30 °C (Fig. 2). The indole NH ($N_1\text{H}$) proton resonance of the sole tryptophan (Trp-58) was identified from its characteristically low-field chemical shift in a spectrum obtained from the protein in water, its through-bond connectivity to the indole CH ($C_2\text{H}$) proton in HOHAHA experiments in water, and its through-space connectivities to both $C_{(2)}\text{H}$ and $C_{(7)}\text{H}$ protons in NOESY experiments in water (Fig. 3). The through-bond connectivities for the ring protons $C_{(4)}\text{H}$, $C_{(5)}\text{H}$, $C_{(6)}\text{H}$ and $C_{(7)}\text{H}$ were identified in DQF-COSY (results not shown) and HOHAHA (Fig. 2) spectra in $^2\text{H}_2\text{O}$, and their order deduced from the identification of $C_{(7)}\text{H}$ in NOESY experiments (Fig. 3) as stated above. Four of the six phenylalanines showed a network

Fig. 1. 500 MHz one-dimensional ^1H -n.m.r. spectra at 20 °C, illustrating the conformational difference between the metal-free and metal-bound forms of CheY, and showing the effect of Mn^{2+} on the proton resonances of CheY1'.

Spectra are from (a) 0.8 mM-metal-free protein, CheY2, obtained after several washes of the native protein, CheY1, with 10 mM-EDTA; (b) 0.8 mM-CheY1; (c) 0.8 mM-CheY2 + 0.8 mM- Mg^{2+} (or CheY1'); (d) 0.8 mM-CheY1' + 0.04 mM- Mn^{2+} . All spectra are from $^2\text{H}_2\text{O}$ -exchanged protein, at pD 6.4. The regions of most significant spectral difference between (a) and (b) are noted with arrows on the chemical shift axis. Resonances that are suppressed significantly by Mn^{2+} binding are marked with arrows in (b).

Table 1. Chemical shifts of assigned resonances of residues close to the metal-binding site

Values are expressed in p.p.m., with reference to DSS, at 30 °C.

Assignment	Chemical shift (p.p.m.)		
	CheY1 (metal-bound)	CheY2 (metal-free)	T87I (metal-free)
Trp-58			
N-H	7.74	7.77	7.77
C _α -H	5.48	5.52	5.52
N ₍₁₎ -H	10.31	10.21	10.12
C ₍₂₎ -H	7.26	7.31	7.25
C ₍₄₎ -H	7.37	7.48	7.48
C ₍₆₎ -H	6.75	6.75	6.75
C ₍₆₎ -H	7.26	7.20	7.12
C ₍₇₎ -H	7.48	7.44	7.44
Thr-87			
C _α -H	5.10	5.16	(missing)
C _β -H	4.10	4.03	(missing)
C _γ -H	0.92	0.92	(missing)
Tyr-106			
N-H	8.40	8.38	8.38
C _α -H	5.89	5.94	6.02
C _{β1} -H	2.37	2.34	2.69
C _{β2} -H	2.73	2.78	2.78
C _{2,6} -H	6.76	6.76	6.72
C _{3,5} -H	6.60	6.60	6.67
Thr(16)			
C _α -H	3.81	3.75	3.75
C _β -H	3.35	3.17	3.17
C _γ -H	1.17	1.13	1.13
X	3.19	3.19	2.98
	1.68	1.68	1.55
	0.57	0.57	0.45

of through-space connectivities among their ring protons, and appear to form a hydrophobic cluster similar to that seen with phenylalanines, 8, 30, 53 and 124 in the crystal structure of the metal-free protein (Volz & Matsumura, 1991). The observed NOESY contacts are summarized in Table 2, along with distances calculated from the X-ray structure. A few of the corresponding NOESY cross-peaks are labelled in Fig. 4. The chemical shifts of these four phenylalanines and the through-space connectivities among them are observed to remain unaffected by metal binding (see Fig. 1, Table 3 and Fig. 4). Thus, the X-ray structure was relied upon to assign this cluster of phenylalanines. The C_β, C_α, and in some cases amide protons of the aromatic residues were identified by their through-space connectivities to ring protons in NOESY experiments in ²H₂O and H₂O. A β-proton resonance of one of the two tyrosines shows a NOESY cross-peak to the amide proton of a phenylalanine. This tyrosine was assigned to be Tyr-51, with through-space connectivity to Phe-53. The corresponding distance in the X-ray structure is 0.29 nm (2.9 Å). Phe-124 was then readily assigned, since its aromatic protons show NOESY connectivities to Phe-53, as predicted by the X-ray structure (see Table 2). The HOHAHA spectra in ²H₂O gave sufficiently well-resolved *f*₁-slices for these two phenylalanines so that the C₍₄₎H resonance could be distinguished from the doubly intense resonances due to the (C₍₂₎H, C₍₆₎H) and (C₍₃₎H, C₍₅₎H) pairs. Cross-peaks of Phe-8 and Phe-30 are clearly observable only at higher temperatures (greater than 30 °C). Phe-8 was assigned using a strong NOESY cross-peak to Phe-124 and a few weaker NOESY cross-peaks to Phe-53. Phe-30 was assigned by its NOESY connectivities to Phe-124 and by the absence of NOESY connectivities to Phe-8 and Phe-53. Phe-53, therefore, is

close to the ring systems of Tyr-51, Phe-124 and Phe-8. This probably explains the large upfield shift observed in the aromatic resonances of Phe-53 [7.01, 6.20, 5.64 p.p.m., compared with the random-coil chemical shift values of 7.39, 7.34, 7.30 p.p.m. (Bundi & Wüthrich, 1979)]. By elimination, the second tyrosine was assigned to be Tyr-106. Sequential assignments could not be obtained for Phe-14 and Phe-11. Table 3 shows the chemical shifts of assigned resonances that remain unaffected by the conformational change upon metal binding.

Identification of residues close to the metal-binding site

Comparison of the aromatic region of HOHAHA spectra in ²H₂O for CheY1' and CheY2 (Fig. 2) shows that the ring system of Trp-58 is particularly affected by the conformational change at the metal-binding site. The resulting changes in chemical shifts of the aromatic resonances of Trp-58 (see Table 1) explain the main difference observed in the one-dimensional spectra in Fig. 1: the tallest peak moving from 7.27 p.p.m. in CheY1 to 7.47 p.p.m. in CheY2. The changes in the chemical shifts of Trp-58 resonances caused by loss of bound metal are further confirmed by the differences in NOESY spectra in 90% (v/v) water for CheY1 and CheY2 (Fig. 3). Changes in the chemical shifts of N₍₁₎H of Trp-58, and its associated nuclear Overhauser enhancement (NOE) cross-peaks to C₍₂₎H and C₍₇₎H clearly indicate a conformational change in at least the side-chain of Trp-58. Chemical shifts of the amide and C_α protons of both Trp-58 and Tyr-106 are also affected to some extent by metal binding (Figs. 2 and 4; Table 1). While such changes in chemical shift may result from the changes in ring-current effects caused by the re-orientation of the Trp-58 side-chain alone, a conformational change involving backbone re-orientation cannot be ruled out. One NOESY cross-peak of particular interest is that showing through-space connectivity between an *m*-proton of Tyr-106 (resonating at 6.60 p.p.m.) and the C₍₄₎H of Trp-58 (resonating at 7.37 p.p.m.) in the metal-bound conformation. This long-range through-space connectivity places Tyr-106 close to the metal-binding site near Trp-58. No NOESY cross-peaks are observed between the ring protons of Tyr-106 and Trp-58 in the metal-free form of the protein. This is consistent with all the observations above, indicating a conformational change in the Trp-58 side-chain. Since the chemical shifts of the ring protons of Tyr-106 remain unaffected by metal binding, it is more difficult to interpret these observations in terms of a metal-induced re-orientation of Tyr-106. The changes in the chemical shifts of the amide, C_α and C_β protons of Tyr-106 may arise from a change in the backbone conformation, or from a change in the ring-current effect due to the re-orientation of Trp-58 alone. Although it is surprising that only one ring in a pair of aromatic rings that are spatially close would exhibit ring-current-induced shifts, it is possible to orient the Tyr-106 ring so that only protons on the Trp-58 ring would exhibit ring-current shift effects. The amide protons of residues Asn-59 and Val-107 are readily identified from the C_αH-N_(i+1)H sequential NOESY cross-peaks, and these appear to remain unaffected by the metal binding (see Fig. 4 and Table 3), indicating that if there is a conformational change at the backbone level, it is fairly localized.

Comparison of the aliphatic region of HOHAHA spectra in ²H₂O for CheY1' and CheY2 (Fig. 5) shows that the chemical shifts of several cross-peaks are affected upon metal binding. Slices along the *f*₁-dimension at 5.10 p.p.m. in CheY1' and 5.16 p.p.m. in CheY2 indicate that a threonine spin system (with a unique connectivity pattern for the C_α, C_β and C_γ protons) is also affected by the conformational change upon metal binding (see Table 1). The X-ray structure shows Thr-87 and Thr-16 to be within 0.6 nm (6 Å) and 1.2 nm (12 Å) respectively, of Asp-57. HOHAHA in ²H₂O of the mutant protein T87I (in which Thr-87

Fig. 2. Aromatic-region two-dimensional HOHAHA spectra at 30 °C (with an isotropic mixing time of 40 ms), showing spin-connectivities for Tyr-51, Phe-53, Trp-58, Tyr-106 and Phe-124

Spectra are from (a) 0.3 mM metal-free T87I mutant CheY; (b) 0.3 mM metal-free wild-type CheY (CheY2); (c) 0.3 mM-CheY2 + 0.5 mM-Mg²⁺ (or CheY1'); (d) 0.3 mM-CheY1' + 0.05 mM-Mn²⁺. In all cases the ²H₂O-exchanged protein was maintained in 50 mM-Tris-d₁₁ buffer at pD 7.0. Comparison of (a) and (b) shows that chemical shifts of Trp-58 and Tyr-106 resonances are altered by the T87I mutation. Comparison of (b) and (c) shows that these same chemical shifts are also affected by Mg²⁺ binding, indicating that Trp-58, Tyr-106 and Thr-87 are all located close to the metal binding site. Note that chemical shifts of Tyr-51, Phe-53 and Phe-124 resonances remain unaffected by the mutation (in a) and the metal binding (in c). Comparison of (c) and (d) shows that resonances of Trp-58 and Tyr-106 (encircled) are suppressed by the binding of Mn²⁺, confirming their proximity to the metal-binding site.

has been replaced by Ile) was used to assign this threonine spin system to Thr-87 (Fig. 5). The aliphatic region of the HOHAHA spectra for T87I and the wild-type CheY are almost superimposable, and the cross-peaks associated with Thr-87 stand out clearly in the spectrum from the wild type, since these cross-peaks are missing in the T87I spectrum. Comparison of the aromatic region shows that the chemical shifts of Trp-58 and Tyr-106 are significantly affected in the T87I mutant spectrum (Table 1 and Fig. 2). This is indirect evidence that Thr-87 is close to both Trp-58 and Tyr-106 in the tertiary structure of CheY in

solution. Since a mutation at residue 87 clearly has structural effects at residues 58 and 106, and hence at the phosphorylation site (at Asp-57), it is expected to affect the functional properties of the protein. This is consistent with the observation that there is a high degree of conservation (Thr or Ser) at this location in the primary structure of several homologous bacterial response regulator proteins (J. B. Stock *et al.*, 1989). Furthermore, the T87I mutant used in this study has been shown to have a pronounced defect in its autophosphatase activity (P. Matsumura & M. I. Simon, unpublished work).

Fig. 3. Low-field region NOESY spectra (at 30 °C, in 90% water/10% $^2\text{H}_2\text{O}$, pH 6.4), showing the change in chemical shifts for the $\text{N}_{(1)}\text{H}$, $\text{C}_{(2)}\text{H}$ and $\text{C}_{(7)}\text{H}$ resonances of Trp-58.

These spectra were recorded with a mixing time of 100 ms. Spectrum (a) is from 0.8 mM-metal-bound CheY1; spectrum (b) is from 0.6 mM-metal-free CheY2.

Table 2. NOESY contacts among the aromatic protons of the cluster of phenylalanines 8, 30, 53 and 124, observed in both metal-free and metal-bound CheY

1 Å = 0.1 nm. Distances between hydrogen atoms were calculated using InsightII, a software package from Biosym Technologies (San Diego, CA, U.S.A.).

NOESY contact	Distance in the crystal structure (Å)
Phe-8 $\text{C}_{(2,6)}\text{-H}$ /Phe-53 $\text{C}_{(2,6)}\text{-H}$	3.0
Phe-8 $\text{C}_{(3,5)}\text{-H}$ /Phe-53 $\text{C}_{(2,6)}\text{-H}$	3.4
Phe-8 $\text{C}_{(3,5)}\text{-H}$ /Phe-53 $\text{C}_{(3,5)}\text{-H}$	3.9
Phe-8 $\text{C}_{(4)}\text{-H}$ /Phe-124 $\text{C}_{(4)}\text{-H}$	2.9
Phe-8 $\text{C}_{(4)}\text{-H}$ /Phe-124 $\text{C}_{(3,5)}\text{-H}$	4.3
Phe-53 $\text{C}_{(3,5)}\text{-H}$ /Phe-124 $\text{C}_{(2,6)}\text{-H}$	3.3
Phe-53 $\text{C}_{(4)}\text{-H}$ /Phe-124 $\text{C}_{(3,5)}\text{-H}$	4.7
Phe-124 $\text{C}_{(3,5)}\text{-H}$ /Phe-30 $\text{C}_{(4)}\text{-H}$	2.6
Phe-124 $\text{C}_{(3,5)}\text{-H}$ /Phe-30 $\text{C}_{(3,5)}\text{-H}$	3.9
Phe-124 $\text{C}_{(4)}\text{-H}$ /Phe-30 $\text{C}_{(3,5)}\text{-H}$	3.9

Examination of the aliphatic region of the HOHAHA spectra in $^2\text{H}_2\text{O}$ (Fig. 5) shows two other spin systems which are significantly affected by metal binding and the T87I mutation. Slices in the f_1 dimension suggest one of these spin systems to be a second threonine residue, its resonances shifting from 3.81, 3.35, 1.17 p.p.m. in CheY1 to 3.75, 3.17, 1.13 p.p.m. in CheY2. A search of the crystal structure shows Thr-16 to be about 1 nm (10 Å) from Asp-13 and about 1.2 nm (12 Å) from Asp-57. Threonines 71, 112 and 115 are more than 1.5 nm (15 Å) and 2 nm (20 Å) distant respectively, from Asp-57 and Asp-13. However, no confident sequential assignment could be obtained for this threonine residue from the n.m.r. data alone. The other spin system, marked as X in Fig. 5 and Table 1, could not be identified completely and remains unassigned. Comparison of the spin-connectivity patterns for X in HOHAHA and

Fig. 4. Aromatic-region NOESY spectra in 90% water, showing a few of the network of NOESY cross-peaks connecting a cluster of phenylalanine residues that remain unaffected on metal binding (see Tables 2 and 3)

Spectra are from: (a) 0.8 mM metal-bound CheY1, pH 6.4 and (b) 0.6 mM metal-free CheY2, pH 6.4. The spectra were recorded at 30 °C with a mixing time of 100 ms. Circles have been used to highlight some of the NOE signals that remain unaffected by the conformational change upon metal binding. The F53/F53 NOESY contact refers to the through-space connectivity observed between Phe-53 $\text{C}_{(2,6)}\text{-H}$ and Phe-53 N-H.

Table 3. Chemical shifts of assigned resonances of residues unaffected by metal binding (expressed in p.p.m., with reference to DSS at 30 °C)

Assignment	Chemical shift (p.p.m.)
Phe-8	
$\text{C}_{(2,6)}\text{-H}$	7.21
$\text{C}_{(3,5)}\text{-H}$	6.89
$\text{C}_{(4)}\text{-H}$	6.78
Phe-30	
$\text{C}_{(2,6)}\text{-H}$	6.82
$\text{C}_{(3,5)}\text{-H}$	7.11
$\text{C}_{(4)}\text{-H}$	6.99
Tyr-51	
$\text{C}_{\beta 1}\text{-H}$	4.07
$\text{C}_{\beta 2}\text{-H}$	3.81
$\text{C}_{(2,6)}\text{-H}$	6.92
$\text{C}_{(3,5)}\text{-H}$	6.69
Phe-53	
N-H	9.19
$\text{C}_{(2,6)}\text{-H}$	7.01
$\text{C}_{(3,5)}\text{-H}$	6.20
$\text{C}_{(4)}\text{-H}$	5.64
Asn-59	
N-H	9.11
$\text{C}_{\alpha}\text{-H}$	4.98
Val-107	
N-H	8.93
$\text{C}_{\alpha}\text{-H}$	4.30
Phe-124	
$\text{C}_{(2,6)}\text{-H}$	6.70
$\text{C}_{(3,5)}\text{-H}$	7.27
$\text{C}_{(4)}\text{-H}$	7.47

Fig. 5. Aliphatic-region two-dimensional-HOHAHA spectra in $^2\text{H}_2\text{O}$ showing the changes in chemical shifts of C_αH and side-chain aliphatic protons

Spectra are from (a) 0.3 mM metal-free T87I mutant CheY; (b) 0.3 mM metal-free wild-type CheY (CheY2); (c) 0.3 mM-CheY2 + 0.5 mM- Mg^{2+} (or CheY1'); (d) 0.3 mM-CheY1' + 0.05 mM- Mn^{2+} . Experimental conditions are as described in Fig. 2. The spin system of Thr-87 has been assigned by comparison of (a) and (b). Cross-peaks suppressed by the binding of Mn^{2+} are encircled in (c) and (d). Comparison of (b), (c) and (d) shows that Thr-87 is close to the metal-binding site: the metal-induced conformational change results in changes in chemical shifts of the Thr-87 resonances; these resonances are also suppressed by Mn^{2+} . Cross-peaks belonging to a second threonine spin system, labelled THR, and an assigned spin system, labelled X, are also seen to be suppressed by Mn^{2+} (see text for tentative assignments for THR and X).

DQFCOSY spectra indicates that X is probably a lysine residue. The three identified resonances of this spin system appear to remain unaffected by metal binding (3.19, 1.68, 0.57 p.p.m.), but undergo a change in chemical shift in the T87I mutant (2.98, 1.55, 0.45 p.p.m.), indicating X to be a residue that is spatially close enough to Thr-87 for its conformation to be affected by the T87I mutation. This, along with the observation that the cross-peaks associated with spin-system X show a suppression effect on Mn^{2+} binding (Fig. 5d), gives further evidence of the proximity of Thr-87 to the metal-binding site in CheY. A search of the crystal structure shows Lys-109 to be within 0.5 nm (5 Å) of both Thr-87 and Asp-57. Overall, comparison of HOHAHA, NOESY and DQFCOSY spectra of CheY1, CheY1' and CheY2 show that only a relatively small number of cross-peaks are affected by metal binding (about 5%), implying that the conformational change is limited to a few residues only.

Titration of fluorescence quenching with added magnesium ions (Lukat *et al.*, 1990) was used to determine K_D of the wild-

type and the T87I mutant forms of CheY2, as described under the Materials and methods section. Both proteins showed an emission maximum at 340 nm, with a half-width of about 56 nm, characteristic of a tryptophan residue that is immobilized at the protein surface and only partially exposed to solvent molecules (Burstein *et al.*, 1973). The emission profiles remained unchanged upon metal binding. Quantum yields for the wild-type and the T87I mutant proteins were 0.16 and 0.11 respectively. Scatchard plots indicate 1 mole of Mg^{2+} bound per mole of protein, with a K_D of about 0.6 mM at 20 °C and pH 7 for both proteins. Since the T87I mutation does not affect the metal binding, it appears that although Thr-87 is close to the metal-binding site, it may not be directly involved in the co-ordination of the metal ion, but may participate in the regulation of the phosphorylated protein via structural modification at the phosphorylation site.

The binding of Mn^{2+} is shown very clearly in e.s.r. experiments (Fig. 6) where the six e.s.r. lines caused by free Mn^{2+} in solution (Fig. 6a) are drastically broadened in the presence of CheY2

Fig. 6. E.s.r. spectra at 22 °C, showing the binding of Mn^{2+} to the metal-free protein, CheY2, and its displacement by Zn^{2+} and Mg^{2+} .

All samples contained 50 mM-bis-tris buffer at pH 7 and 0.3 mM- Mn^{2+} . In addition, (b), (c) and (d) contained 0.3 mM-CheY2. (b) and (c) also contained 0.4 mM- Zn^{2+} and Mg^{2+} respectively. Spectra were recorded on a Varian E-4 e.s.r. spectrometer with a variable temperature unit.

(Fig. 6d). Addition of Zn^{2+} (Fig. 6b) or Mg^{2+} (Fig. 6c) appears to displace the protein-bound Mn^{2+} partially, thus releasing some free Mn^{2+} (approx. 10 %) which increases the peak height of the e.s.r. signal. However, the peak height could never be regained completely, indicating that a large fraction of Mn^{2+} remained bound even in the presence of excess Mg^{2+} or Zn^{2+} . One possible explanation for this observation would be that there are more Mn^{2+} binding sites than Mg^{2+} or Zn^{2+} binding sites per CheY molecule. This was checked by adding excess Mn^{2+} to a known concentration of protein and removing the free metal ions by several washes in an Amicon ultrafiltration cell. The protein appears to bind Mn^{2+} tightly enough for bound Mn^{2+} to be retained after several washes. Evaluation of the observed Mn^{2+} concentration by double integration of the resulting e.s.r. signal showed that the binding stoichiometry was 1:1, consistent with the results from fluorescence quenching experiments with Mg^{2+} . Lukat *et al.* (1990) have shown that K_D values for Mn^{2+} , Zn^{2+} and Mg^{2+} are 0.047 mM, 0.097 mM and 0.45 mM respectively for CheY from *Salmonella typhimurium*. It is therefore more likely that competitive binding and relative exchange rates control the fraction of Mn^{2+} displaced by other bivalent metal ions in the e.s.r. experiments described above.

N.m.r. experiments were also performed using Mn^{2+} to observe paramagnetic metal-induced enhancement of relaxation and hence probe the environment of the bound metal. Addition of low concentrations of Mn^{2+} to the CheY1' form of the protein suppresses several resonance lines in one-dimensional n.m.r. (Fig. 1d), specifically those corresponding to the Trp-58 residue, indicated to be affected by the conformational change. The doublet in the aromatic region corresponding to Tyr-106 is significantly suppressed at higher concentrations of Mn^{2+} than those required for suppression of the Trp-58 resonances, indicating that the metal-binding site is closer to Trp-58 than Tyr-106.

This suppression effect is more clearly seen in two-dimensional-HOHAHA experiments in $^2\text{H}_2\text{O}$ in the presence of Mn^{2+} (see Figs. 2d and 5d). Cross-peaks corresponding to the Trp-58 spin system are drastically suppressed (almost eliminated from the two-dimensional map) owing to the proximity of Trp-58 to the bound Mn^{2+} . The suppression of cross-peaks corresponding to Tyr-106 aromatic resonances are less obvious at the low Mn^{2+} concentrations used to generate these spectra, but the axial peak

corresponding to the Tyr-106 C_αH resonance at 5.89 p.p.m. is seen to be suppressed, as is the axial peak corresponding to the Trp-58 C_αH resonance at 5.48 p.p.m. Note that the specific suppression of Trp-58 cross-peaks by Mn^{2+} leaves an unambiguous assignment for the cross-peaks of Phe-124, with chemical shifts close to those of the Trp-58 aromatic resonances (Fig. 2d). Several cross-peaks in the aliphatic region are also suppressed by Mn^{2+} , including those assigned to Thr-87 (Fig. 5d). A sharp aliphatic peak at 1.98 p.p.m. shows a dramatic suppression on Mn^{2+} binding (Fig. 1d). Examination of f_1 slices at this p.p.m. in the HOHAHA spectra indicates that this resonance most probably belongs to a system with no through-bond connectivity to any other spins, and is tentatively assigned to the ϵ -methyl of a methionine residue. In the crystal structure, the ϵ -methyl carbon of Met-60 is 0.64 nm (6.4 Å) and 0.74 nm (7.4 Å) respectively, from the carboxylic carbons of Asp-57 and Asp-13, while the corresponding distances for Met-17 and Met-85 are 0.7 nm (7.0 Å), 1.13 nm (11.3 Å) and 0.79 nm (7.9 Å), 1.3 nm (13.0 Å). There is a high frequency of occurrence for Met at position 60 in homologous response regulator proteins (J. B. Stock *et al.*, 1989).

Comparison of two-dimensional-HOHAHA spectra in $^2\text{H}_2\text{O}$ (Figs. 2 and 5) also shows that the majority of the cross-peaks that are significantly shifted by the bivalent-metal-induced conformational change are also significantly suppressed by Mn^{2+} . Therefore it appears that the conformational change is not only limited to a small number of residues, but is restricted to residues close to the metal-binding site. Without complete assignment of all cross-peaks, we cannot rule out the possibility of minor changes at other locations in the protein, farther from the metal-binding site. It is to be noted, however, that the cross-peaks assigned to residues 8, 30, 51, 53 and 124, which form a hydrophobic cluster about 2 nm (20 Å) from the phosphorylation site, remain unaffected by the conformational change induced by bivalent metal binding. This conclusion is not weakened by the fact that the crystal structure was relied upon for the sequential assignment of the four phenylalanines in this cluster. The cluster is well defined by a network of inter-residue NOESY cross-peaks which remain unaffected by the conformational change upon metal binding. The spin systems in the cluster are definitely identifiable as a tyrosine and four phenylalanines. Hence, regardless of the sequential assignment of the four phenylalanines in this cluster, the added information from the Mn^{2+} -binding experiments enable us to conclude that this hydrophobic region, far from the metal-binding site, is left unaffected by the metal-induced conformational change.

The suppression of cross-peaks by Mn^{2+} in the two-dimensional HOHAHA experiments was used to estimate distances from the bound manganese to a few of the residues at the metal-binding site. Volume integrals of cross-peaks were obtained using the FELIX software package. When spectra from the control experiments were compared with spectra from experiments using Mn^{2+} , ratios of volume integrals of the unaffected cross-peaks between the two experiments (cross-peaks of Phe-124 and Tyr-51, for instance) were found to be close to 1 (± 0.1). Therefore, even this simplistic treatment of the two-dimensional HOHAHA intensities should give reasonably good estimates of relative distances from the bound metal ion, without requiring further calibration. The volume integrals of the suppressed cross-peaks relative to those in the control experiments were used to estimate the separation between the bound paramagnetic ion and the residues close to the metal-binding site (see the Materials and methods section). The effective correlation time, τ_c , was determined using the relationship

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_b} \quad (4)$$

Fig. 7. Stereo diagram of the immediate region around the active site of metal-free CheY

Residues included in the view are those that are inferred to be close to the metal-binding site from a triangulation of distances estimated from two-dimensional n.m.r. cross-peak suppression on Mn^{2+} binding. Both conformations of the Tyr-106 ring predicted by X-ray are shown. Side-chains of some of the residues (Thr-16, Ser-56, Val-86 and Ala-88) have been omitted for clarity. N.m.r. indicates metal-induced conformational changes for Trp-58, Thr-87 and possibly Tyr-106.

where τ_r is the rotational correlation time for the Mn^{2+} bound to CheY, and τ_s and τ_b are the electronic spin-relaxation time and the bound-state lifetime for Mn^{2+} respectively. Since the e.p.r. spectra (Fig. 6) indicate that the protein-bound Mn^{2+} is essentially immobilized relative to the protein, the rotational correlation time of the CheY molecule is a good estimation for τ_r , with a value of $(0.5-1) \times 10^{-8}$ s [similar to that of lysozyme (Schmidt & Kuntz, 1984)]. τ_s for Mn^{2+} is $10^{-8}-10^{-9}$ s (Bertini *et al.*, 1989). τ_b is determined to be about 10^{-7} s, assuming diffusion-controlled binding (Cantor & Schimmel, 1980). Under these assumptions, τ_c is estimated to be about 2.5×10^{-9} s and $f(\tau_c)$ is about 10^{-8} s. The fraction of binding sites, B , occupied by Mn^{2+} per protein molecule is about 0.15 under the conditions of our HOHAHA experiments. The uncertainty in r is estimated to be about 35%, arising from a factor of 6 uncertainty in the product $[f(\tau_c)B/\pi\Delta\nu_p]$ in eqn. (3). The measured volume integrals then lead to the following estimates for distances from the bound Mn^{2+} : Thr-87, < 0.8 nm (8 Å); Trp-58 (ring protons), < 0.8 nm (8 Å); Trp-58 ($C_\alpha H$), 0.5–1.1 nm (5–11 Å); Tyr-106 ($C_\alpha H$), 0.6–1.2 nm (6–12 Å); Tyr-106 (ring protons), 0.7–1.4 nm (7–14 Å); X (unassigned), 0.6–1.2 nm (6–12 Å); and Thr(16), 0.7–1.4 nm (7–14 Å).

The X-ray crystal structure of CheY from *S. typhimurium* (*St*-CheY) has been resolved to 0.27 nm (2.7 Å) (A. M. Stock *et al.*, 1989). An acidic pocket formed by Asp-12, Asp-13 and Asp-57, on the same face of the molecule and at one end of a central parallel β -sheet, was suggested as the phosphorylation site. The crystal structure of *Ec*-CheY has also been determined (Volz & Matsumura, 1991) and refined to 0.17 nm (1.7 Å) (*St*-CheY has Phe, Ile and Ser at positions 51, 54 and 76 respectively, replacing Tyr, Val and Gly at these positions in the primary sequence of *Ec*-CheY, and the crystal structure of *St*-CheY was determined using heavy-atom derivatives of the S56C mutant protein). In the high-resolution structure of *Ec*-CheY, the side-chain of Tyr-106 has two possible values for χ_1 , so that the aromatic ring system of Tyr-106 occupies two sites with equal probability; one site is more accessible to solvent and further from Asp-57 than the other [about 1 nm (10 Å) and 1.5 nm (15 Å) from Asp-57 respectively], while Tyr-51 is more than 1.7 nm (17 Å) from Asp-57. Our n.m.r. results show that one of the two tyrosines (Tyr-106) may be involved in the conformational change caused by

metal binding, while the other (Tyr-51) remains unaffected. Resonances of Tyr-106 are also suppressed in Mn^{2+} -binding experiments (while those of Tyr-51 are not), confirming the proximity of Tyr-106 to the metal-binding region close to Asp-57. The n.m.r. data, therefore, are consistent with that conformation of Tyr-106 in the single crystal which corresponds to a shorter distance between Tyr-106 and Asp-57. In this conformation, protons of Tyr-106 are 0.3–0.7 nm (3–7 Å) from protons of Trp-58 in the high-resolution structure of the metal-free protein. The observed NOE between Tyr-106 and Trp-58 in the metal-bound form of the protein, and its absence in the metal-free protein, indicates that the relative conformation of these two residues is different in the two forms of the protein. It may also imply that the conformational change upon metal binding brings these two residues closer, and hence brings Tyr-106 closer to the phosphorylation site in the metal-bound form of the protein. However, the relative suppression of resonances by Mn^{2+} , and distances estimated using cross-peak suppression in two-dimensional n.m.r., show that Trp-85 (and hence Asp-57) is closer to the metal-binding site than Tyr-106.

A systematic study of the effects of mutations at aspartyl residues 12, 13 and 57 points to Asp-57 as the normal site of phosphorylation in CheY (Bourret *et al.*, 1990). In our n.m.r. study, the cross-peaks in the two-dimensional HOHAHA spectra that are most significantly affected by Mn^{2+} binding belong to Trp-58. Trp-58 is also highly affected by the conformational change that occurs on metal binding, which implicates the phosphorylation site at Asp-57 as being directly involved in the metal binding and the consequent conformational change. It is quite likely that the negative charges of residues like Asp-13 and Asp-57 play an important role in both metal binding and phosphotransfer activity, as inferred by Lukat *et al.* (1990). Aspartic acid residues at these locations appear to be highly conserved in phosphorylation-activated response regulators homologous to CheY. Our n.m.r. observations indicate that Thr-87, Tyr-106 and a methionine (possibly Met-60) may also play a critical role in defining the structure of the active site. Examination of the CheY crystal structure shows that the distances calculated using cross-peak suppression in two-dimensional n.m.r. from the bound Mn^{2+} to Trp-58, Thr-87, Tyr-103 and

Thr-16 are consistent with the proposed metal-binding site involving Asp-57 and Asp-13 (Lukat *et al.*, 1991), assuming metal-induced conformational changes are small. Residues inferred to be close to the metal-binding site from a triangulation of these estimated distances are shown in Fig. 7.

It has been shown that Mg^{2+} is required for both phosphorylation and dephosphorylation (Hess *et al.*, 1988a,b; Lukat *et al.*, 1990). The correlation between bivalent-cation binding and localized effects on Trp-58, Thr-87 and Tyr-106, as demonstrated by our n.m.r. data, indicates that this region may be important to the phosphorylation biochemistry. The functional importance of Thr-87 has been established: the T87I mutant used in the n.m.r. study is defective in its autophosphatase activity (P. Matsumura & M. I. Simon, unpublished work). Further, a K109R mutant has been shown to have a reduced autophosphatase activity also (Lukat *et al.*, 1991). Lys-109 is adjacent to Thr-87 in the crystal structure of CheY (Volz & Matsumura, 1991). It is therefore very possible that the bivalent-metal-induced conformational change of CheY reported here forms part of its phosphorylation-activated switching mechanism which regulates signal transduction in bacterial chemotaxis.

We noted earlier that the native metal-bound form, CheY1 (in which the metal ion is bound *in vivo*), could not be regenerated completely by addition of bivalent metal ions *in vitro* to the metal-free form, CheY2. Small differences in conformation are indicated by the differences observed in the n.m.r. spectra of CheY1 and CheY1', particularly in the chemical shifts of the Trp-58 and Tyr-106 resonances and the relative intensity of the sharp aliphatic resonance tentatively assigned to the ϵ -methyl of a methionine residue (see Fig. 1). Metal binding affinity, however, appears to be quite different. The bound metal ion (excluding Mn^{2+}) in CheY1' may be removed completely by two to three washes in an Amicon ultrafiltration cell, thereby converting CheY1' back into CheY2. This is consistent with the observed K_D values of 0.6 mM for Mg^{2+} and 0.045 mM for Mn^{2+} (Lukat *et al.*, 1990). However, the conformation of CheY1 remains unchanged even after dialysis to remove Tris, or several washes in an Amicon ultrafiltration cell, indicating that the metal is bound more tightly in CheY1. Examination of the filtrates by atomic absorption spectroscopy (AA) shows that no metal is lost in the washes. Excess EDTA must be used to extract the bound metal from CheY1. Estimation of the concentration of bound metal in CheY1 by AA shows that the binding stoichiometry is 1:1, as in CheY1'. These two metal-bound forms of the protein also differ in their interaction with phosphates, as described in the following paper (Kar *et al.*, 1992). We have no explanation as yet for this difference. However, we have eliminated protein degradation as a cause. CheY1', obtained from freshly prepared CheY2 by the addition of bivalent metals, shows phosphate-binding properties that are distinctly different from those of freshly prepared CheY1 (Kar *et al.*, 1992). Also, CheY1', CheY2 and CheY1 are all stable at low temperatures (0–10 °C) for over 1 month, and their respective metal- and phosphate-binding properties remain unchanged over this time period. Our attempts to obtain CheY1 from CheY2 *in vitro* (for instance, by urea denaturation of CheY2, followed by renaturation in the presence of excess bivalent metal ions) have been unsuccessful. If an additional cofactor is involved, we do not have any evidence of it so far.

In summary, one-dimensional and two-dimensional n.m.r., in conjunction with paramagnetic-metal-ion-induced relaxation, has been used to investigate the observed binding of bivalent metals to CheY. Assignments of the aromatic residues alone permit probing of two distinct regions of the protein: (i) the region around the phosphorylation site at Asp-57, at one end of the central parallel β -sheet in CheY (using Trp-58 and Tyr-106 resonances), and (ii) the hydrophobic region formed by a cluster

of four phenylalanines and a tyrosine (8, 30, 53, 124 and 51), at the opposite end of the β -sheet, about 2 nm (20 Å) from the phosphorylation site. It is shown that the metal ion binds close to the phosphorylation site at Asp-57 on the protein, causing a specific local conformational change that may be central to the role played by CheY in bacterial signal transduction. Residues Trp-58, Thr-87 and Tyr-106 and a methionine (possibly Met-60) are shown to be close to the metal-binding site and are involved in the metal-induced conformational change. These results may prove helpful in the design of site-directed mutation experiments to elucidate the molecular mechanisms involved and the role of CheY as a phosphorylation-activated switch in bacterial chemotaxis.

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