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Kinetics of CheY phosphorylation by small molecule phosphodonors

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Received 20 July 1999

Abstract The chemotaxis response regulator CheY can acquire phosphoryl groups either from its associated autophosphorylating protein kinase, CheA, or from small phosphodonor molecules such as acetyl phosphate. We report a stopped-flow kinetic analysis of CheY phosphorylation by acetyl phosphate. The results show that CheY has a very low affinity for this phosphodonor ($K_s \gg 0.1$ M), consistent with the conclusion that, whereas CheY provides catalytic functions for the phosphotransfer reaction, the CheA kinase may act simply to increase the effective phosphodonor concentration at the CheY active site.
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Key words: Two-component system; Phosphotransfer; Chemotaxis

1. Introduction

Signal transduction pathways in microorganisms often involve a conserved histidine-aspartate phosphotransfer chemistry mediated by two interacting proteins: histidine protein kinases (HPKs) and their associated response regulators [1]. HPKs are generally composed of two conserved domains, a catalytic or C-domain [2,3] and a phosphoaccepting histidine or H-domain [4,5]. The C-domain binds ATP and catalyzes the transfer of its γ-phosphoryl group to the N-3 position of a histidine side chain in the H-domain. The phosphorylated H-domains serve as phosphodonors for the phosphorylation of an invariant aspartate residue in cognate response regulator proteins. The site of aspartate phosphorylation is part of a highly conserved Mg^{2+} binding pocket [6–8]. The His to Asp phosphotransfer chemistry is dependent on metal binding to this site and it has been suggested that the reaction is catalyzed by the response regulator with the HPK primarily functioning in the generation of substrate phosphohistidine groups [9,10]. A catalytic role for the response regulators was established by the observation that small molecules such as acetyl phosphate (AcP), phosphoramidate (Pam), carbamoyl phosphate or phosphoimidazole can act as phosphodonors in place of phosphorylated histidines in HPK H-domains [9,11–18].

Recent reports have raised the possibility that the H-domain also plays an essential role in catalyzing the phosphotransfer reaction [19,20]. The results indicate that the observed constant, k_{obs}, for phosphorylation of the chemotaxis response regulator CheY by small molecule phosphodonors is several orders of magnitude less than that observed for phosphorylation by the cognate HPK, phospho-CheA, ~ 0.1 s^{-1} versus 800 s^{-1}. Such a 10^4-fold rate enhancement, if it was true, would clearly implicate the CheA protein as a catalyst for the transfer of phosphoryl groups to CheY.

The kinetics of CheY phosphorylation by small molecules has generally been analyzed by measuring a phosphorylation-associated quenching of intrinsic fluorescence of the single tryptophan residue in CheY, Trp_{58}, which is located adjacent to the site of aspartate phosphorylation, Asp_{57} [9]. This approach was used to measure the rate of CheY phosphorylation by AcP, Pam and phosphoimidazoles [19]. The results appeared to give similar maximum k_{obs} values for all phosphodonor molecules tested (~ 0.1 s^{-1}), whereas the apparent K_ms for different phosphodonor substrates varied from about 0.6 to 3 mM. But the kinetic experiments on which these conclusions were based were seriously flawed in that the reported instrumental mixing times were similar to the maximal reaction rates that were observed. This raised the possibility that the small values obtained for maximum k_{obs} derive from slow mixing times rather than slow maximal rates of phosphotransfer.

Here, we report a re-examination of the kinetics of CheY phosphorylation by small molecule phosphodonors. A stopped-flow apparatus was used to insure adequate temporal resolution. The results clearly show that, in contrast to the previous findings, CheY phosphorylation by AcP or Pam is a first order reaction with respect to the phosphodonor concentration with no indication of substrate saturation at concentrations up to 100 mM. Moreover, in contrast to previously published conclusions [20], high ionic strength does not lead to an increase in affinity for AcP. Under all conditions, CheY clearly has such a low affinity for AcP and Pam that it is not possible to estimate the maximum k_{obs}. There is no evidence that CheA serves any other function than to provide, through specific protein-protein interactions, a high local concentration of phosphohistidine at the CheY active site.

2. Materials and methods

2.1. Materials

AcP (potassium, lithium) was purchased from Sigma. The potassium salt of Pam was synthesized by the method of Sheridan et al. [21]. The *Salmonella typhimurium* CheY protein was purified as described previously [22].

2.2. Stopped-flow kinetic measurements

Stopped-flow kinetic experiments were performed in 100 mM Tris-HCl, 20 mM Mg^{2+}, pH 7.0, buffer at 20°C unless specified otherwise. In the experiments with a constant ionic strength of 0.45 M, KCl was added in the AcP solutions in order to have a final ionic strength of 0.45 M. For the experiments at 1.65 M, both CheY and AcP solutions were prepared in the above Tris-Mg^{2+} buffer containing KCl so that

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Abbreviations: AcP, acetyl phosphate; HPK, histidine protein kinase; Pam, Phosphoramidate

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PII: S0014-5793(99)01057-1

the ionic strength was set at 1.65 M before mixing. A Hi-Tech scientific SF-61DX2 microvolume stopped-flow reaction analyzer equipped with a high intensity xenon lamp was used to follow CheY fluorescence (excitation 295 nm, 2.0 mm excitation slit, 320 nm cutoff emission filter). Fluorescence was recorded for up to 200 s after mixing 2.0 μM CheY with an equal volume of AcP or Pam to give the indicated final concentrations of phosphodonor. The instrument has a mixing time less than 2.0 ms. Control experiments where CheY was mixed with buffer alone showed no significant change in fluorescence. The data obtained at each phosphodonor concentration were analyzed using the Kinetasyst 2 software provided by Hi-Tech scientific by fitting a single exponential plus linear function to the average of three or four individual time courses, each consisting of 510 points. If the linear component was omitted from the analysis and the data fitted to a simple exponential, there were no significant differences in the estimated values for the observed rate constants, k_{obs}.

The kinetics of CheY phosphorylation by AcP were analyzed in terms of the following chemistry [9]:

$$\mathrm{CheY} + \mathrm{AcP} \underset{k_{-1}}{\overset{k_{+1}}{\Leftrightarrow}} \mathrm{CheY{\cdot}AcP} \xrightarrow{k_{+2}} \mathrm{CheY \sim P} + \mathrm{AC} \xrightarrow{k_{+3}} \mathrm{CheY} + \mathrm{Pi}.$$

Assuming that [AcP] > [CheY], $k_{+2} \gg k_{-2}$, $k_{+3} \gg k_{-3}$ and $k_{-1} \gg k_{+2}$, the observed rate constant, k_{obs}, for CheY phosphorylation can be expressed as [23]:

$$k_{obs} = k_{+3} + \frac{k_{+2}\cdot[\mathrm{AcP}]}{(k_{-1}/k_{+1}) + [\mathrm{AcP}]} \quad (1)$$

According to this model, the rate of CheY phosphorylation should increase with the AcP concentration until it reaches a plateau saturation value equal to (k_{+2}+k_{+3}), where k_{+2} is the rate of CheY phosphorylation by AcP bound at the active site and k_{+3} is the pseudo first order rate constant for CheY ~ P dephosphorylation. The equilibrium dissociation constant of the CheY-AcP enzyme-substrate complex, K_s, corresponds to k_{-1}/k_{+1}.

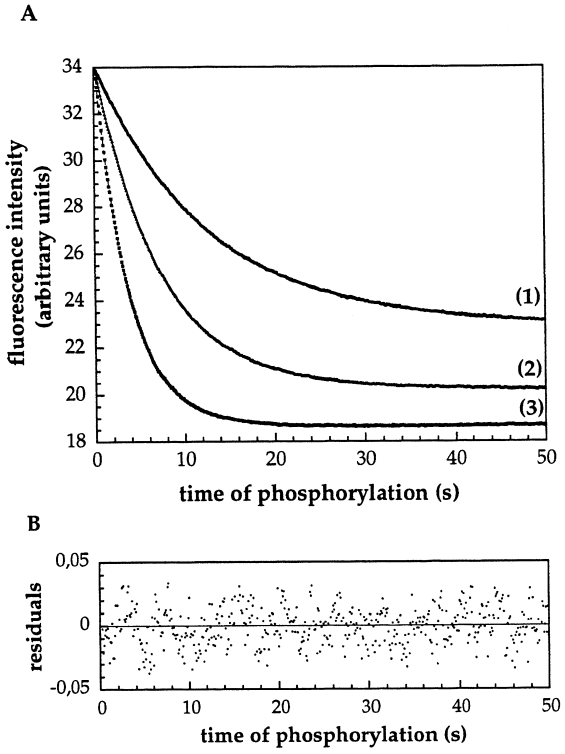

Fig. 1. Kinetics of CheY phosphorylation by AcP. A: CheY (final concentration 1.0 μM) was rapidly mixed with AcP to give the indicated final concentrations of phosphodonor. The decrease in fluorescence as a function of time was monitored as described in Section 2. Representative traces are shown for 10 (1), 25 (2) and 75 (3) mM AcP. The solid lines represent the best single exponential fit for each curve. B: Residuals for 25 mM AcP (2) are shown under the fit.

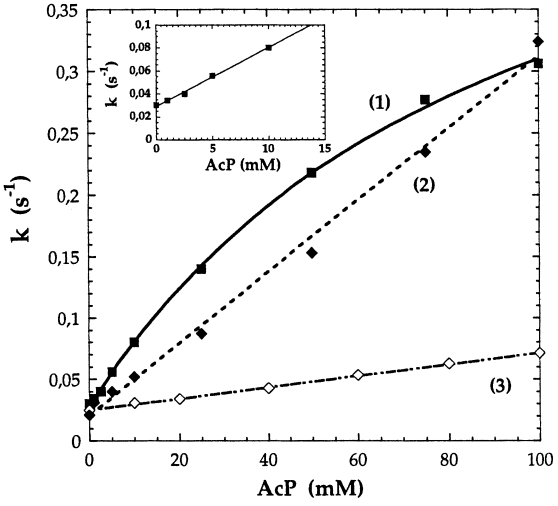

Fig. 2. Pre-steady-state kinetics of CheY phosphorylation by AcP under different conditions of ionic strength. Stopped-flow experiments with AcP in 100 mM Tris-HCl, 20 mM Mg^{2+}, pH 7.0, at 20°C (■), at a constant ionic strength of 0.45 M (♦) or at a constant ionic strength of 1.6 M (◇). The ionic strength was maintained constant by addition of appropriate amounts of KCl. The curves are best fits to a hyperbolic saturation (curve 1) or a linear function (curves 2 and 3). The *Y*-intercept corresponds to k_{+3}, the first order rate constant for P-CheY dephosphorylation).

3. Results

3.1. Kinetics of CheY phosphorylation by small phosphodonor molecules

The kinetics of CheY phosphorylation were measured by monitoring the phosphorylation-associated decrease in tryptophan fluorescence using a stopped-flow apparatus with a resolution in the ms–10^3 s range. Equal volumes of CheY (final concentration 1.0 μM) were mixed with AcP (final concentrations indicated). At all AcP concentrations tested, there was an exponential decrease in CheY fluorescence over a period of 1–3 min (Fig. 1). The values of the corresponding rate constants, k_{obs}, were plotted as a function of the AcP concentration (Fig. 2, curve 1). The results indicated a first order dependence on AcP at concentrations below 15 mM (Fig. 2, insert). At higher concentrations of AcP, a slight but significant deviation from linearity was observed as if CheY was becoming saturated with AcP. The data gave a good fit (solid curve) to Eq. 1 (Section 2), with $K_s = 94.6 \pm 9.0$ mM and maximum $k_{obs} = 0.55 \pm 0.03$ s^{-1}. The rate of dephosphorylation of phospho-CheY, which corresponds to the *Y*-intercept on a plot of k_{obs} versus the phosphodonor concentration, was 0.030 ± 0.002 s^{-1}, consistent with previously determined values [20,24,25]. The K_s value obtained in these experiments was about 10-fold higher than those reported by Silversmith et al. [19], but this could have been due to differences in temperature and buffer conditions. We therefore repeated the experiment with AcP under precisely the conditions that were previously used and found a significantly higher K_s than previously reported (72 ± 9 mM versus 11 mM [19], data not shown). Similarly higher K_s values were obtained with Pam (data not shown). These discrepancies can readily be explained by the instrumental limitations in the previously reported experiments.

These results indicate that CheY has higher maximal veloc-

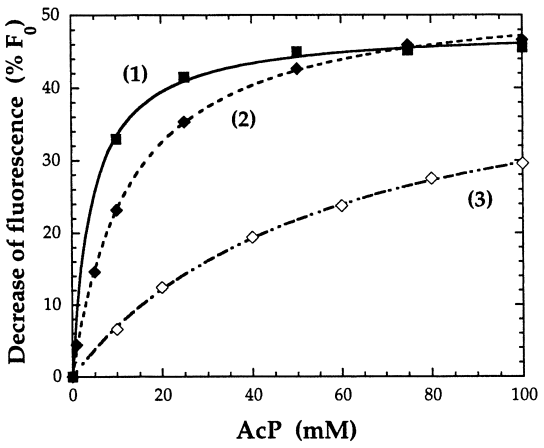

Fig. 3. Steady-state kinetics of CheY phosphorylation by AcP. A: The amplitudes of maximum fluorescence decrease calculated as a percentage of the initial fluorescence value and plotted as a function of AcP concentrations. Data were from the stopped-flow experiments shown in Fig. 2: 100 mM Tris-HCl, 20 mM Mg^{2+}, pH 7.0, at 20°C (■, curve 1), in 100 mM Tris-HCl, 20 mM Mg^{2+}, pH 7.0, at 20°C adjusted to a constant ionic strength of either 0.45 M (♦, curve 2) or 1.65 M (◇, curve 3) with KCl. The curves are best fits to a hyperbolic saturation function.

ities of phosphorylation and significantly lower affinities for AcP and Pam than previously reported [19]. Nevertheless, the maximal rates estimated from the data presented in Fig. 1 are still less than 0.1% of the rates that have been estimated for phosphotransfer from phospho-CheA to CheY. It should be noted, however, that the maximum rate estimates derived from this type of analysis depend critically on the assumption that slight deviations from linearity at high AcP and Pam concentrations are caused by substrate saturation. Subsequent experiments have shown that this is not, in fact, the case. It has recently been shown that elevated salt inhibits CheY phosphorylation by small molecules [20], which could easily account for the apparent saturation of CheY by concentrations of AcP and Pam in the 100 mM range.

To test this possibility, we examined the dependence of the rate of phosphotransfer on AcP concentrations under conditions of constant ionic strength. Results obtained under approximately physiological conditions of ionic strength, 0.45 M, showed a strictly first order dependence on AcP (Fig. 2, curve 2). This finding strongly supports the idea that small molecule phosphodonors have a very low affinity for CheY. In much higher ionic strength medium, 1.65 M, the rate of CheY phosphorylation by AcP was substantially reduced, but there is no evidence whatsoever for CheY saturation (Fig. 2, curve 3).

3.2. Steady-state fluorescence quenching

Whereas the rate of CheY phosphorylation is linearly dependent on the concentration of AcP, the rate of dephosphorylation remains constant. Steady-state levels of CheY phosphorylation therefore increase with increasing AcP and these increases lead to a progressive increase in fluorescence quenching. This effect saturates when essentially all of the CheY that is present has been converted to the phosphorylated form (Fig. 3). Half-maximal phosphorylation was observed at 4.4 ± 0.3 mM AcP in 100 mM Tris, at 12.8 ± 0.4 mM AcP in 450 mM ionic strength buffer and at 56.5 ± 2.5 mM AcP in 1.65 M ionic strength buffer. Because increased salt inhibits the phosphorylation reaction, but has little affect on the rate of dephosphorylation (Fig. 2, [20]), increases in salt cause substantial increases in the concentrations of phosphodonors required to achieve half-maximal steady-state levels of CheY phosphorylation.

4. Discussion

The transient kinetics of CheY phosphorylation by small molecule phosphodonors such as AcP can be described by a three step model, a fast bimolecular reaction followed by phosphotransfer and slow dephosphorylation. The lack of saturation of the initial rate at elevated phosphodonor concentrations indicates that the affinity of AcP (or Pam) for CheY is extremely low. The results of the steady-state experiments are in agreement with this conclusion. The active site of CheY is presumably not designed to bind AcP or Pam. Rather, phosphorylation by these agents appears to be a consequence of CheY phosphotransfer chemistry.

The ratio k_{+2}/K_s is given by the slope of the rate of CheY phosphorylation as a function of [AcP] (Fig. 2). This ratio corresponds to the catalytic efficiency of the enzyme, i.e. the rate constant of the bimolecular reaction between enzyme and substrate. From the value of $\sim 3\ M^{-1}\ s^{-1}$ for k_{+2}/K_s at physiological ionic strength, since $K_s \gg 100$ mM, $k_{+2} \gg 0.3\ s^{-1}$. The rate constant of phosphotransfer from phospho-CheA to CheY appears to be 650–800 s^{-1} [20,25]. If k_{+2} for CheY phosphorylation by AcP has a comparable value, say 500 s^{-1}, then, K_s would have to be about 170 M. Phosphoimidazole, which is a better analogue for phosphohistidine than AcP or Pam, appears to exhibit about a 6-fold higher rate at comparable concentrations [19]. Assuming that k_{+2} for this substrate is comparable to the phosphohistidine, K_s for the imidazole phosphate bound to CheY would be about 30 M.

It seems likely that the function of the HPKs in the transfer reaction is primarily to bind to their cognate response regulators so as to increase the local concentration of phosphohistidine groups at the phosphoaccepting active site. Certainly, the phosphotransfer specificity of two-component systems must derive from the molecular fit between cognate kinases and regulators. The cross talk that is generally observed between heterologous kinase-regulator pairs and the phosphodonor abilities of small molecules appear to reflect that latent enzymatic activity of the response regulator domain. This activity probably only becomes relevant, however, when it functions in conjunction with specific binding interactions.

Acknowledgements: We warmly thank Dr Roger Goody (Dortmund, Germany), Dr Michael Levit (Princeton, USA) and Dr Fabrice Agou (Institut Pasteur, France) for stimulating discussions and Benoit Schneider for his friendly help. This work was supported by Grants from the NIH to J.B.S. (R01GM57773) and from the Association de la Recherche contre le Cancer and the Ligue Nationale contre le Cancer to M.V.

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