Temperature-Induced Switching of the Bacterial Flagellar Motor

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ABSTRACT Chemotaxis signaling proteins normally control the direction of rotation of the flagellar motor of *Escherichia coli*. In their absence, a wild-type motor spins exclusively counterclockwise. Although the signaling pathway is well defined, relatively little is known about switching, the mechanism that enables the motor to change direction. We found that switching occurs in the absence of signaling proteins when cells are cooled to temperatures below about 10°C. The forward rate constant (for counterclockwise to clockwise, CCW to CW, switching) increases and the reverse rate constant (for CW to CCW switching) decreases as the temperature is lowered. At about −2°C, most motors spin exclusively CW. At temperatures for which reversals are frequent enough to generate a sizable data set, both CCW and CW interval distributions appear to be exponential. From the rate constants we computed equilibrium constants and standard free energy changes, and from the temperature dependence of the standard free energy changes we determined standard enthalpy and entropy changes. Using transition-state theory, we also calculated the activation free energy, enthalpy, and entropy. We conclude that the CW state is preferred at very low temperatures and that it is relatively more highly bonded and restricted than the CCW state.

INTRODUCTION

The bacterium *Escherichia coli* executes a three-dimensional random walk. It swims along a smooth path (runs), moves erratically for a brief time (tumbles), and then runs again, choosing a new direction approximately at random. In a spatial gradient of a chemical attractant, runs that happen to carry a cell in a favorable direction are lengthened (Berg and Brown, 1972). Runs occur when the motors that drive flagellar filaments spin counterclockwise (CCW) when viewed from outside the bacterium, and tumbles occur when they spin clockwise (CW); therefore, the control of the direction of flagellar rotation underlies the chemotactic response (Larsen et al., 1974). The rotation of a flagellar motor can be observed by tethering a cell to a glass surface by a single flagellar filament: the cell body spins alternately CW and CCW (Silverman and Simon, 1974). The motor behaves as a two-state system, with constant probabilities per unit time of transitions between CW and CCW states, and exponential CW and CCW interval distributions (Block et al., 1982). The behavior of the system can be described in terms of forward and reverse rate constants for switching. When cells are exposed to an exponential ramp of a chemical attractant—this increases receptor occupancy linearly with time—the forward (CCW to CW) rate constant decreases whereas the reverse (CW to CCW) rate constant increases, and motors spend a larger fraction of their time spinning CCW. The change in the forward rate constant is larger than that in the reverse rate constant (Block et al., 1983). These changes are mediated by the response regulator CheY, which when phosphorylated effects CW rotation (Hess et al., 1987, 1988; Oosawa et al., 1988; Wylie et al., 1988). Cells that are deleted for CheY, or its kinase CheA, spin exclusively CCW (Parkinson, 1977; Ravid and Eisenbach, 1984; Wolfe et al., 1987). Several reviews on bacterial chemotaxis have appeared recently (Barak and Eisenbach, 1995; Blair, 1995; Macnab, 1995; Stock and Mowbray, 1995; Eisenbach, 1996; Macnab, 1996).

How does CheY-phosphate control the direction of flagellar rotation? Does the motor necessarily spin CW when a certain number of molecules are bound, or does binding simply stabilize the CW state at the expense of the CCW state (i.e., shift the standard free energies)? The latter seems more likely, given that there are mutants in proteins of the flagellar switch (FliG, FliM, or FliN) that allow the motor to switch in the absence of CheY (e.g., Ravid and Eisenbach, 1984). We show here that one can change the standard free energy difference of these states simply by changing the temperature. At low temperatures, the CW state has the lower standard free energy. Whether this is a direct effect of temperature on the switch or an indirect effect due to other changes in the physiological state of the cell remains to be determined. However, a direct effect seems likely, because loss of protonmotive force (Khan and Macnab, 1980) or ATP (Shioi et al., 1982) enhances CCW rotation.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: Apeizon Products, Ltd. (London, England), Apiezon grease M; Difco Laboratories, Inc. (Detroit, MI), bactotryptone; Fisher Scientific (Pittsburgh, PA), sodium chloride, enzyme grade, sodium phosphate dibasic anhydrous, sodium phosphate monobasic, monohydrate, both American Chemical Society certified; Sigma Chemical Co. (St. Louis, MO), dimethylsulfoxide, dipotassium salt

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of EDTA, kanamycin monosulfate. Other chemicals were reagent grade. Water was deionized (18 MΩ-cm) and filtered (0.2 μm).

Cells

We studied *E. coli* strain HCB437, deleted for genes encoding transducers and chemotaxis signaling proteins Δ(tsr)*7021*, Δ(*trg*)*100* *zbd*::Tn5, Δ(*cheA-cheZ*)*2209*, *met F159*(Am) (Wolfe et al., 1987). Cultures were grown and prepared for tethering as described earlier (Berg and Turner, 1993), with the following exceptions. Frozen aliquots of bacteria were thawed and grown for 4.5 h at 30°C. Tethering buffer was 10 mM sodium phosphate (pH 7), 0.1 mM EDTA, 67 mM sodium chloride. Filaments were sheared from cells by passing the cell suspension back and forth between two syringes 65 times. The final cell density was 5 times that in the growth medium.

Tethering

Cells were tethered to glass as described earlier (Berg and Tedesco, 1975), with the following exceptions. The glass coverslip tethering surface was cleaned with 95% ethanol saturated with potassium hydroxide. The clean coverslip was supported by two other coverslips attached to a microscope slide with Apeizon M grease. Cells suspended in tethering buffer with anti-filament antibody at a titer of 1/500 were added between the slide and coverslip (coverslip down). Tethering occurred within 45 min at room temperature. Untethered cells were washed away with tethering buffer, and the preparation was stored at room temperature.

Data acquisition

The rotation of tethered cells was recorded with a microscope (Nikon Optiphot-2) equipped with a 40× bright phase objective, a 2.5× projection lens, a CCD camera (Hamamatsu type 77 with c2400 controller), a time generator (homemade), and a VCR recorder (Panasonic AG6730). The videotape was analyzed by eye on a 9-inch monitor (Sony PVM96) using the frame-by-frame search mode of an editing-style recorder (Panasonic AG6300). When a cell changed its direction of rotation, the time recorded on the frame was noted, together with the number of revolutions from the previous reversal. The smallest time interval recorded was 0.033 s, and the least distance was 0.1 revolution.

Temperature control

The microscope was equipped with a calibrated temperature control that cooled both the objective and the stage, using a water-cooled Peltier element and two thermistors (Kahn and Berg, 1983), one near the Peltier element in the control feedback circuit and the other next to the slide. A separate temperature calibration was performed using a smaller thermistor at the tethering site, with silicon oil substituted for the tethering buffer. All thermistors were calibrated with a mercury thermometer certified by the National Institute of Standards and Technology. The temperature measured at the tethering site was used in all calculations. The Peltier element was cooled with water from a refrigerated circulator (Lauda Brinkman RM6) set to 2.5°C. The microscope stage, objective, and cooling element were housed in a plexiglass box containing a dessicant to eliminate convective air currents and condensation. Temperatures set with the control feedback circuit stabilized within 1 min, and video recording was begun 3 min later.

RESULTS

We used a strain of *Escherichia coli* (HCB437) that lacks chemotaxis signaling proteins, including the response regulator CheY, but which forms intact motor-switch complexes. At room temperature, tethered cells of this strain spin exclusively CCW. Unexpectedly, we found that motor reversals occurred at temperatures below about 10°C. Fig. 1 *A* shows the reversal frequency for six tethered cells from −2°C to 12°C. Fig. 1 *B* is the CCW bias for the same cells. The bias varied from 1 (100% CCW rotation) at about 12°C to 0 (0% CCW rotation, or 100% CW rotation) at about −2°C. The reversal frequency at each temperature was determined as shown in Fig. 2 *A*. At temperatures at which switching was frequent, distributions of the time intervals between reversals were exponential (Fig. 2 *B*). This justified the simple two-state description of the system used below for analysis of the data. As the temperature was lowered, the reversal frequency increased and later decreased (Fig. 1 *A*). However, the fraction of time that the cells spun CCW steadily declined (Fig. 1 *B*). Forward (CCW to CW) and reverse (CW to CCW) rate constants (Figs. 3 *A* and *B*) were calculated from the reversal frequency and the fraction of time spent in each state. As the temperature was lowered, the speed of the motor decreased (Fig. 4).

We calculated the activation energies (Figs. 5 *A* and *B*), enthalpies, and entropies (Figs. 6 *A* and *B*) for the forward and reverse transitions for each cell from tran-

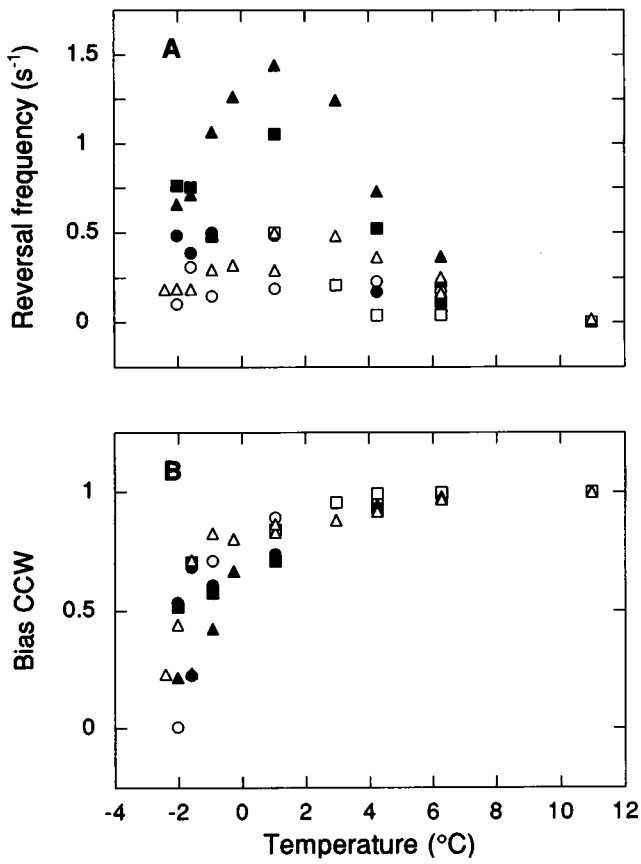

FIGURE 1 (*A*) The reversal frequency and (*B*) the bias CCW (time CCW/total time) as a function of temperature for each cell: 1 (△), 2 (▲), 3 (□), 4 (●), 5 (○), 6 (■). Cell 1 has more than one data point for each temperature, because data were collected both as the temperature was lowered and raised. Reversal frequencies were computed as described in Fig. 2 *A*.

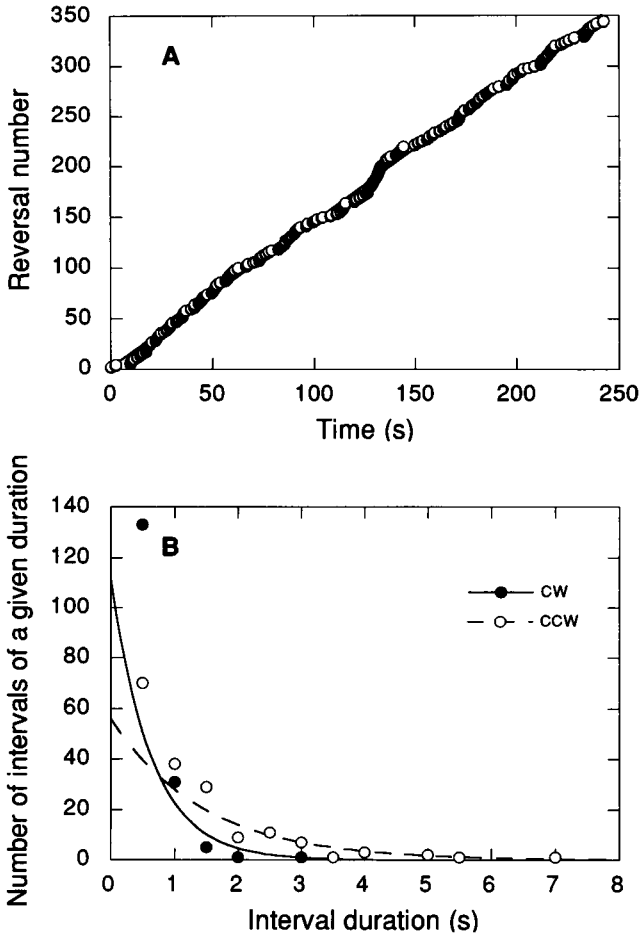

FIGURE 2 An example of the kind of data acquired at each temperature for all cells (cell 2 at 0.89°C). (*A*) Each reversal of the motor was assigned a number (in sequence) and plotted at the time when it occurred. A linear least-squares regression yields a correlation coefficient r equal to 0.99. The slope of this line fit is the reversal frequency. (*B*) The time intervals between reversals were binned according to their duration: ●, CW rotation; ○, CCW rotation. For each data set, a least-squares fit to an exponential curve yields a correlation coefficient r greater than 0.97.

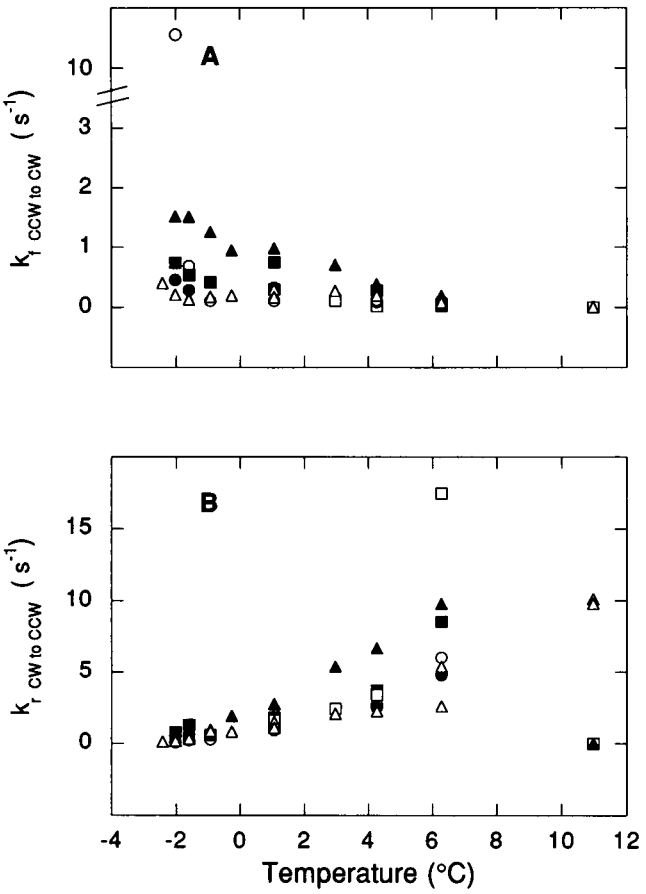

FIGURE 3 (*A*) Forward (k_f, CCW to CW) and (*B*) reverse (k_r, CW to CCW) rate constants plotted as a function of temperature for each cell. The symbols are the same as in Fig. 1.

Data analysis

Assuming the switch to be in equilibrium and representative of an ensemble when followed at high time resolution over

sition state theory (see below). The standard free energy change was calculated from the equilibrium constant at each temperature (Fig. 5 *C*), and the standard enthalpy and entropy changes were calculated from the standard free energy change as a function of temperature (Fig. 6 *C*) (see below). In Fig. 7 we present free energies (means for all cells) as a series of energy barrier diagrams at each temperature. Even though the values vary from cell to cell, the overall trend is the same (Table 1). The standard enthalpy and entropy changes and the forward activation enthalpies and entropies are all negative; the reverse activation enthalpies and entropies are all positive. We conclude that the CW state is more constrained (or highly bonded) than the CCW state. The standard free energy change is fairly linear with temperature over the range −3 kJ/mol to 15 kJ/mol. At low temperatures, the CW state is favored over the CCW state.

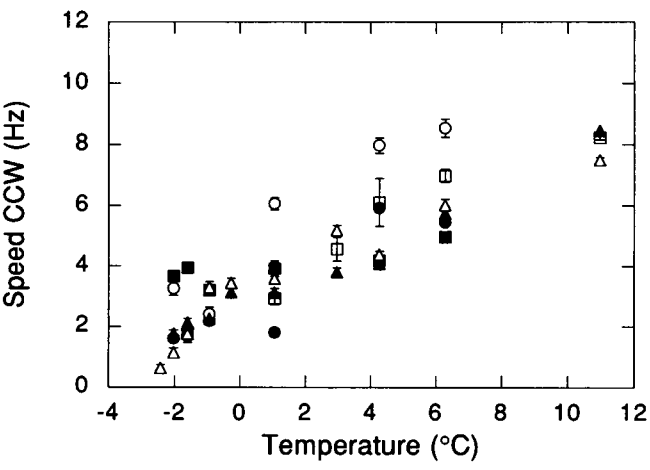

FIGURE 4 The mean rotation speed for all CCW intervals plotted as a function of temperature for all cells. The data were corrected for changes in the viscosity of the buffer due to temperature. The symbols are the same as in Fig. 1. Error bars are standard errors.

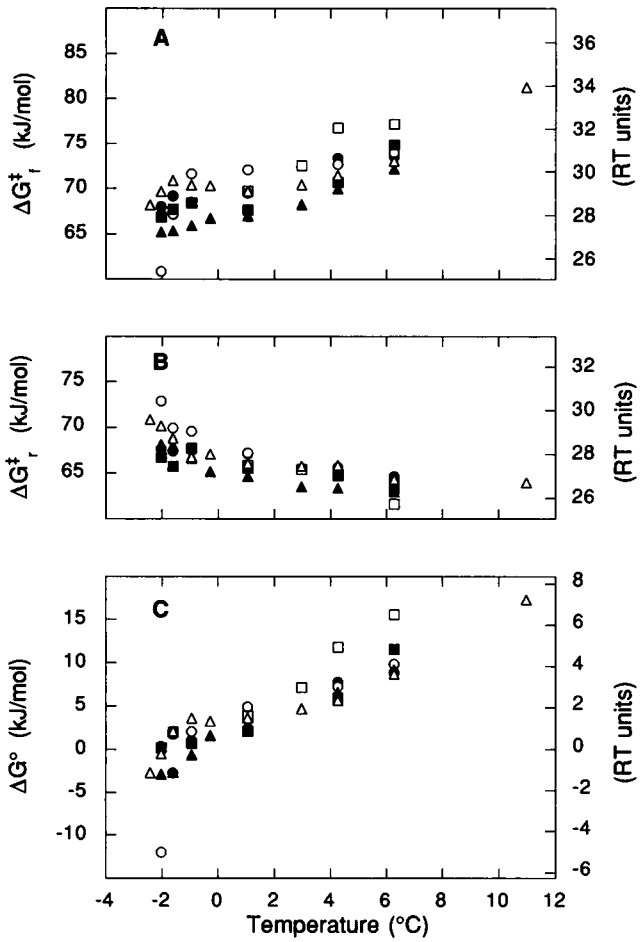

FIGURE 5 (*A*) The activation energy for the forward transition (CCW to CW) shown as a function of temperature for each cell. (*B*) The activation energy for the reverse transition (CW to CCW). (*C*) The standard free energy of the transition. The symbols are the same as in Fig. 1. *RT* was evaluated at 15°C.

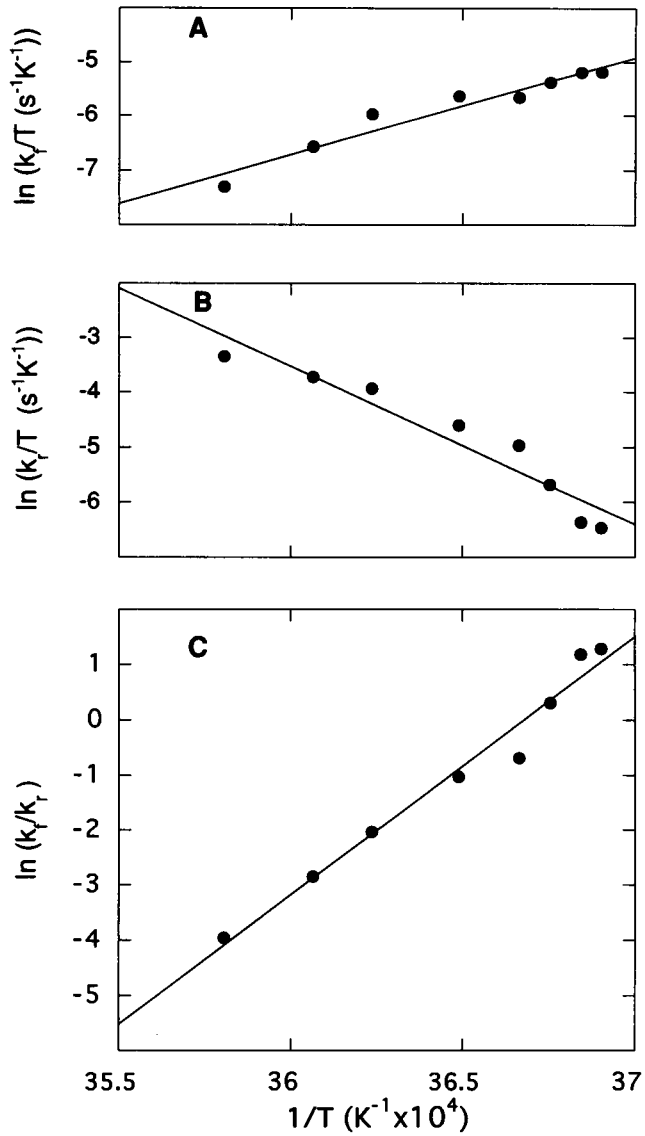

FIGURE 6 Examples of least-squares line fits (cell 2). (*A*) The temperature dependence of k_f. $\Delta H_f^{\ddagger}$ and $\Delta S_f^{\ddagger}$ were evaluated from the line fit slope and intercept, respectively. (*B*) The same plot as in *A* for k_r. (*C*) The same plot as in *A* for the equilibrium constant $K = k_f/k_r$.

a few minutes, we fitted the observations to a two-state model (Block et al., 1982):

$$\mathrm{CCW} \underset{k_r}{\overset{k_f}{\Leftrightarrow}} \mathrm{CW}. \tag{1}$$

Here k_f and k_r are the forward and reverse rate constants (transition probabilities per unit time), respectively. This system should be characterized by an exponential distribution of time intervals in each state, as was found to be the case. At each temperature (T) the two rate constants (k_f and k_r) and hence the equilibrium constant (K) and the standard free energy change of the transition ($\Delta G°$) could be evaluated from the video record. This was done as follows. We denote the fraction of time spent in the CCW or CW states by f_{CCW} or f_{CW}, respectively, and the reversal frequency by ρ. These quantities are readily obtained from the record (see Fig. 1 and Fig. 2). It follows that

$$f_{CCW} = k_r/(k_f + k_r), \quad f_{CW} = k_f/(k_f + k_r) = 1 - f_{CCW},$$

and consequently

$$\rho = f_{CCW}k_f + f_{CW}k_r = 2k_f k_r/(k_f + k_r). \tag{2}$$

Hence the rate constants are given by

$$k_f = \rho/2f_{CCW}, \quad k_r = \rho/2f_{CW} \tag{3}$$

and the equilibrium constant by

$$K = f_{CW}/f_{CCW}. \tag{4}$$

Accordingly,

$$\Delta G° = -RT \ln(f_{CW}/f_{CCW}). \tag{5}$$

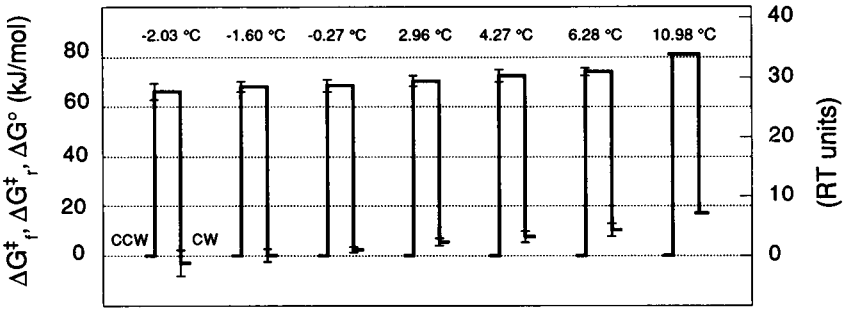

FIGURE 7 Differences in free energy between the CCW, transition, and CW states, shown as a function of temperature for all cells. Error bars are standard deviations. *RT* was evaluated at 15°C.

From the dependence of these parameters on T, we evaluated the standard enthalpy ($\Delta H°$) and entropy ($\Delta S°$) changes by plotting the relationship (Fig. 6 *C*)

$$\ln(f_{CW}/f_{CCW}) = \Delta S°/R - \Delta H°/RT. \quad (6)$$

The activation energies ($\Delta G_f^‡$, $\Delta G_r^‡$), enthalpies ($\Delta H_f^‡$, $\Delta H_r^‡$), and entropies ($\Delta S_f^‡$, $\Delta S_r^‡$) for the forward and reverse transitions were obtained from transition state theory (reviewed in Eyring and Urry, 1965; for a similar approach to channel conductance see McLarnon and Wang, 1991, and Zanello and Barrantes, 1994). Considering the forward transition, this gives

$$k_f = \kappa(kT/h)\exp(-\Delta G_f^‡/RT) = \kappa(kT/h)\exp(-\Delta H_f^‡/RT)\exp(\Delta S_f^‡/R), \quad (7)$$

where κ, the "transmission coefficient," may be taken as 1 for most biological reactions (Eyring and Urry, 1965), and k and h are the Boltzmann and Planck constants, respectively. (Similar expressions hold for the reverse reaction.) Accordingly, we calculated $\Delta G_f^‡$ directly from Eq. 7, and $\Delta H_f^‡$ and $\Delta S_f^‡$ were evaluated from the temperature dependence of k_f by plotting the relationship (Fig. 6 *A*)

$$\ln(k_f/T) = \ln(k/h) + \Delta S_f^‡/R - \Delta H_f^‡/RT. \quad (8)$$

(The equivalent relationship for the reverse reaction is plotted in Fig. 6 *B*.) It is seen from Eq. 7 that $\Delta G° = \Delta G_f^‡ - \Delta G_r^‡$, and similarly for $\Delta H°$ and $\Delta S°$.

The six cells tested showed completely consistent behavior. The values of $\Delta H°$, $\Delta S°$, $\Delta H_f^‡$, and $\Delta S_f^‡$ were all negative; those of $\Delta H_r^‡$ and $\Delta S_r^‡$ were all positive (Table 1). The activation energies $\Delta G_f^‡$ and $\Delta G_r^‡$ decreased and increased, respectively, with decreasing T (Fig. 5, *A* and *B*).

DISCUSSION

The main conclusion of this study, in which the response regulator CheY is not involved, is that the CW state of the switch has a lower standard free energy at low temperatures than the CCW state. Although a direct effect of temperature seems likely, as mentioned earlier, we cannot exclude an indirect effect due to changes in the physiological state of the cell. A further conclusion is that over the temperature range studied, the standard enthalpy and entropy changes for the forward (CCW to CW) transition are essentially constant and negative. This implies that hydrophobic associations cannot play a major role in the forward transition, as the standard entropy change would then be positive, although they may well be involved in the reverse transition. The CW conformation appears to be more restricted and is probably more highly bonded. Any proposed mechanism must be consistent with these observations, even though there is no straightforward method of obtaining a mechanism from kinetic data.

The mean standard enthalpy and entropy changes for the forward transition are, respectively, about −400 kJ/mol and

TABLE 1 Enthalpy and entropy changes

Cell	$\Delta H_f^‡$ (kJ/mol)	$\Delta S_f^‡$ (kJ/K · mol)	*r* fit	$\Delta H_r^‡$ (kJ/mol)	$\Delta S_r^‡$ (kJ/K · mol)	*r* fit	$\Delta H°$ (kJ/mol)	$\Delta S°$ (kJ/K · mol)	*r* fit
1	−131	−0.737	0.77	195	0.465	0.93	−326	−1.201	0.95
2	−150	−0.793	0.97	240	0.636	0.96	−389	−1.428	0.99
3	−350	−1.533	0.92	282	0.786	0.94	−632	−2.319	0.99
4	−121	−0.698	0.92	163	0.353	0.99	−284	−1.050	0.98
5	−226	−1.077	0.64	314	0.897	0.97	−540	−2.273	0.84
6	−152	−0.806	0.85	173	0.391	0.94	−325	−1.197	0.94
Mean	−188	−0.941		228	0.588		−416	−1.578	
SD	87	0.320		61	0.222		139	0.569	

For each cell, plots were constructed according to Eq. 8 from the forward and reverse rate constants at each temperature. Activation enthalpy and entropy were determined from line fits to those plots. These are the data in columns 1, 2 and 4, 5, with the correlation coefficient of the line fit in columns 3 and 6, respectively. For each cell the standard free energy, enthalpy, and entropy changes were calculated from the equilibrium constant at each temperature (see text). These are the data in columns 7 and 8, with the correlation coefficient of the line fit in column 9.

-1.5 kJ/K · mol (see Table 1). Representative values for protein folding at room temperature are -200 kJ/mol and -0.5 kJ/K · mol (Kunz et al., 1995; Privalov, 1992). Our measured values are on this order of magnitude but are significantly higher numerically; it should be born in mind, however, that we are discussing a conformational change of unknown extent in an assemblage of many protein molecules. The marked decrease in entropy due to the forward transition is probably the consequence of the disruption of hydrophobic interactions, offset at low temperature by the enthalpy change arising from electrostatic and van der Waals interactions as well as hydrogen bonding. If we take the number of copies of the FliM protein, the subunit most extensively involved in switching, to be about 25 (Macnab, 1995), and the magnitude of the hydrophobic effect as -0.6 J/K · mol per Å^2 of nonpolar surface exposed to water (Privalov, 1992), we then arrive at an estimate of 100 Å^2 nonpolar surface exposed per FliM subunit during the forward transition.

We now consider some implications of our data with respect to the action of CheY. It is interesting to note that if our linear plot of the standard free energy change ($\Delta G°$) versus temperature (Fig. 5 *C*) is extrapolated to room temperature (23°C), assuming this is justified, then we reach a value of about +40 kJ/mol (roughly the energy of two hydrogen bonds). This is an independent confirmation of the fact that CheY does not function as an enzyme, i.e., it does not simply lower the activation energy of the transition, because the increase in standard free energy is so high that even lowering the barrier would not result in any substantial population of the CW state. So it must be the case that the complex after binding is characterized by a much reduced standard free energy in the CW state relative to the CCW state. Hence dissociation of CheY-P must be accompanied by a return to the CCW state.

According to Stock et al. (1995), if we consider the activation of a response regulator such as CheY by phosphorylation, a conformational change in the regulator must occur with a $\Delta G°$ of about -40 kJ/mol (or even higher numerically). From our result it appears that binding of CheY-P at room temperature somehow lowers the standard free energy of the CW conformation of the switch (relative to the CCW conformation) by at least 40 kJ/mol. The $\Delta G°$ of switching becomes sufficiently small, or even negative, that it becomes a highly probable event. One possible scenario is that after binding of CheY-P to switch subunit FliM in the CCW state (Eisenbach, 1996; Macnab, 1995), with a binding energy of, say, no more than 1 or 2 kJ/mol (see below), the transition to CW involves a transient reorientation of CheY-P. This might well be a transient partial or complete reversal of the original activating conformational change of CheY, perhaps with transient dephosphorylation. (Recently, though, Macnab (1995) expressed the view that there does not seem to be any need to invoke switch-mediated dephosphorylation of CheY-P.) The transient reorientation of CheY-P could be followed by a joint conformational change of the bound complex with a $\Delta G°$ of the required magnitude. This idea implies that binding of CheY-P enormously increases the probability of a forward transition, and release of CheY-P is almost certainly followed by a backward transition. Such a scheme is very close to the model proposed by Macnab (1995). It is also consistent with one of the possible four-state models of normal switch action put forward by Kuo and Koshland (1989) on the basis of kinetic studies with cells containing CheY:

$$\begin{array}{ccc} & K_1 & \\ \mathrm{CCW} & \Leftrightarrow & \mathrm{CW} \\ K_3 \Updownarrow & & \Updownarrow K_4 \\ \mathrm{CCW \cdot CheY\text{-}P} & \Leftrightarrow & \mathrm{CW \cdot CheY\text{-}P} \\ & K_2 & \end{array}$$

(clearly two further states could be added for the binding of nonphosphorylated CheY). If we associate four equilibrium constants with this scheme as shown, two for the transitions from CCW to CW, and two for the relevant binding reactions, we then have

$$K_1K_4 = K_2K_3. \tag{9}$$

However, it is probable that several CheY-P molecules binding to different FliM molecules act cooperatively to cause CW rotation. Supposing that N such associations occur successively and independently, with individual binding constants K_3' and K_4', we have $K_3 = (K_3')^N$ and $K_4 = (K_4')^N$. It is plausible to assume that at room temperature K_2 has a value similar to that of K_1 at the lowest temperatures at which we measured $\Delta G°$. It also is plausible to assume that K_3 cannot be high, because Bren et al. (1996) have shown that CheZ, which probably acts as a regulator (Barak and Eisenbach, 1995), mediates dephosphorylation of free rather than switch bound CheY-P. Hence estimating that $K_1 \approx 10^{-7}$ (from the value of $\Delta G°$ at room temperature inferred above) and $K_2 \approx 1$, we find that $K_4/K_3 \approx 10^7$. (For individual binding events, $K_4'/K_3' \approx 10^{7/N}$.) This strengthens our supposition that complete release of CheY-P in the CW state is rare, but in any event is followed by a reversion to the CCW state.

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