Regulation of CFTR ion channel gating by MgATP

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Abstract Single channel currents of wild-type CFTR reconstituted in lipid bilayers were recorded to study the temperature dependence of channel gating between +20°C and +40°C. The opening of the channel was highly temperature dependent and required an activation energy of about 100 kJ/mol. Closing of the channel was only weakly temperature dependent with an activation energy close to that of diffusion in water. We found no significant difference in the free energy between the open and closed states. Most of the excess energy needed to activate channel opening is used to diminish the entropy of the open state. This structural reorganization is initiated by ATP binding followed by interconversion to the open channel structure as the CFTR-ATP-Mg complex passes to the transition state for hydrolysis. The energy of the CFTR-ATP-Mg interaction in the transition state is responsible for the CFTR ion channel opening rather than the energy of ATP hydrolysis. Channel closing is a diffusion limited process and does not require additional ATP binding.

Key words: CFTR; Gating kinetics; Temperature dependence; Arrhenius plot; Gating thermodynamics; ATP hydrolysis

1. Introduction

Cystic fibrosis (CF) is a lethal genetic disease characterized by defective transepithelial Cl^- transport [1,2]. This disease is caused by mutations in a single gene. The affected gene was identified and the gene product named the cystic fibrosis transmembrane conductance regulator (CFTR) [3,4]. According to the sequence, CFTR is a member of the ABC transporter super-family [5]. In contrast to other family members, CFTR is responsible for the passive transport of chloride ions down an electrochemical gradient and operates as a typical ion channel. The CFTR ion channel must be phosphorylated by PKA and also requires hydrolysable nucleoside triphosphate on the cytoplasmic side to be opened [6,7]. However, not all studies find that ATP hydrolysis is an absolute requirement [8,9]. Several models for CFTR channel gating have been considered [10,11]. Each of these were explicitly or implicitly based on the assumption that energy of ATP hydrolysis is used to regulate ion channel gating. This is an unusual way to regulate ion channel gating and requires an unknown mechanism of energy transduction. According to their functional similarities, all known ion channels may be classified in two main families: voltage-gated channels and ligand-gated channels [12]. For the voltage-gated family, transitions between open and closed states are thermal-driven processes and the electrical field defines the final distribution between states. For the ligand-gated family, binding of the ligand changes the configuration of the closed state to enable thermal-driven transitions between open and closed states to occur. In both cases thermal equilibrium between states should be established. If ATP hydrolysis is required for the opening and/or closing of the CFTR ion channel, then thermal equilibrium between open and closed states should not exist. Here we present data indicating that the open and closed states of the CFTR ion channel are in thermal equilibrium with MgATP operating as a ligand. The binding energy of MgATP in the CFTR-MgATP complex plays a major role in stabilizing the open channel structure. In this model we suppose structural coupling between the formation of the transition state structure for ATP hydrolysis and the open channel structure rather than energetic coupling between them. Additional ATP binding is not required to close the channel. We speculate that ATP hydrolysis is required to make the gating process reversible. A preliminary report of this work has appeared previously in abstract form [13].

2. Materials and methods

2.1. Isolation of microsomal membrane vesicles from CHO cells

Microsomal vesicles from CHO cells stably expressing CFTR [14] were isolated by a protocol developed earlier for CHO cell fractionation [15] and similar to that employed more recently by Gunderson and Kopito [16] for studies of CFTR in planar lipid bilayer. Prior to use, vesicles at a protein concentration of 1–3 mg/ml in 10 mM HEPES, pH 7.2 containing 5 mM $MgCl_2$ and 250 mM sucrose were phosphorylated by addition of ATP to 2 mM and catalytic subunit of protein kinase A to 100 units/ml for a 15 min incubation at room temperature.

2.2. Single channel measurements

Planar lipid bilayers were painted onto a 0.2 mm hole drilled in a Teflon cup using a phospholipid solution in *n*-decane containing a 2:1 mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (20 mg/ml). The lipid bilayer separated 1.0 ml of solution in the Teflon cup (*cis* side) from 4.0 ml of solution in an outer glass chamber (*trans* side). Both chambers were magnetically stirred and thermostated. Heating and temperature control were established using a Temperature Control System TC2BIP (Cell MicroControls). Electrical contact with the solutions was provided by Ag/AgCl electrodes through agar bridges filled with 0.5 M KCl. The membrane potential difference was measured as the difference between *cis* and *trans* side potentials. The *trans* side was grounded and electrical measurements of the single channel current were performed under voltage clamp conditions using an Axopatch 200B (Axon Instruments) amplifier. The output signal was filtered with an 8-pole low pass Bessel filter LPBF-48DG (NPI Electronic GmbH) with cut off frequency of 50 Hz and recorded on magnetic tape by using a VR-10B (Instrutech Corp.) digital data recorder and a Sony SLV-440 VCR. For data analysis the signal was digitized (Digidata 1200; Axon Instruments) with a sampling rate of 500 Hz and analyzed using pCLAMP 6.0 (Axon Instruments) software. Origin 4.0 (Microcal) software was used to fit all points histograms by multi-peak Gaussians. Membrane vesicles were added as a concentrated stock solution to the *cis* side to obtain a final total protein concentration of 10–15 μg/ml. All measurements were done in symmetrical salt solutions containing, in mM: 300 Tris-HCl, 2 MgCl, 1 EGTA,

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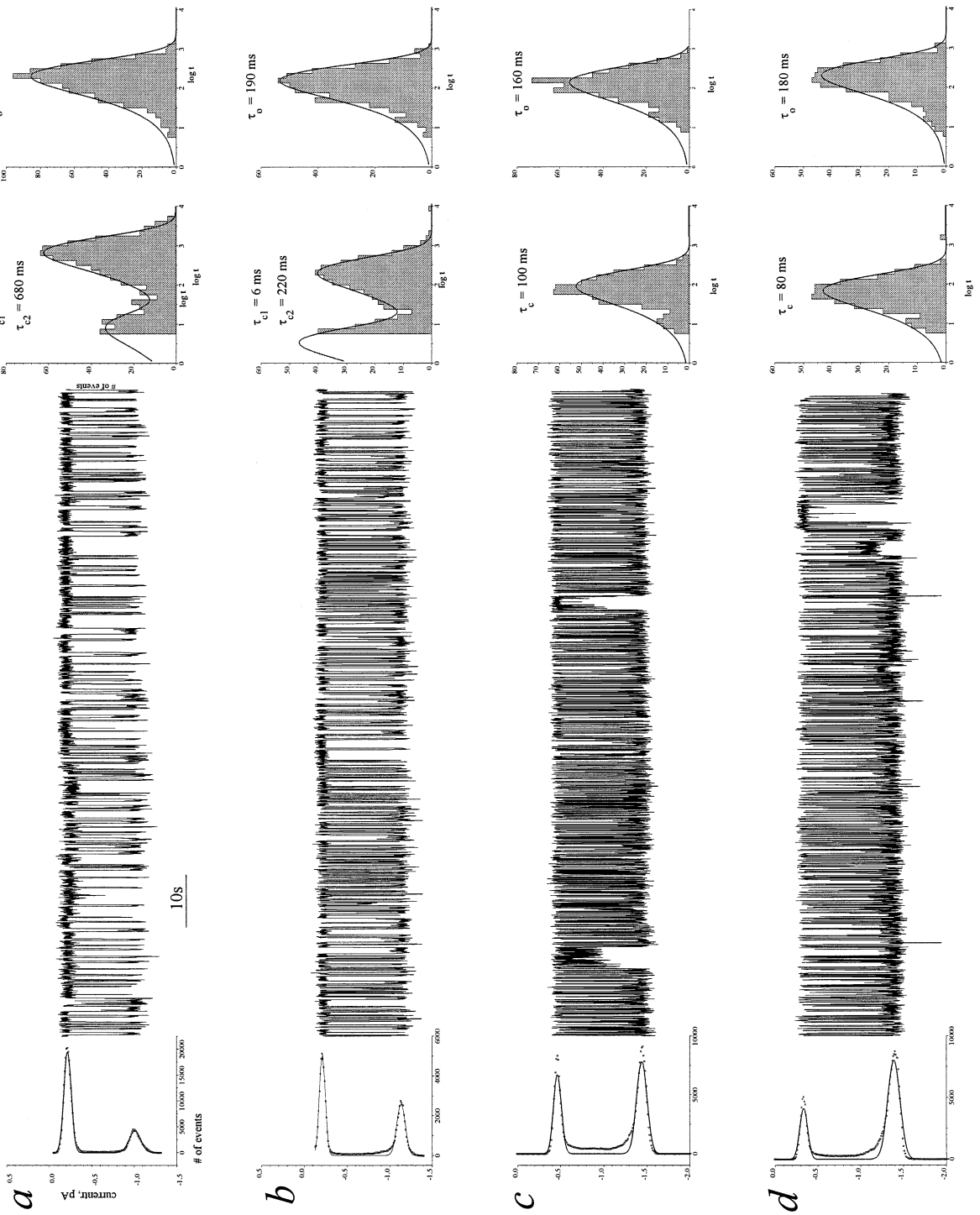

Fig. 1. CFTR single channel kinetics at different temperatures. CFTR single channel recorded at different temperatures. a: +25°C, b: +30°C, c: +35°C, d: +40°C. All points histograms are shown on the left. Areas under these peaks on the all points histograms were used to calculate P_o. Two min portions of the single channel records which were used to prepare dwell-time histograms are shown in the center. Dwell-time histograms for closed and open state, sequentially, are shown on the right. The numerical values shown above the dwell-time histograms are time constants for the closed and open states calculated for these particular experiments. Ten min single channel records were used to prepare the dwell-time histograms for a: +25°C and b: +30°C and 2 min records for c: +35°C and d: +40°C.

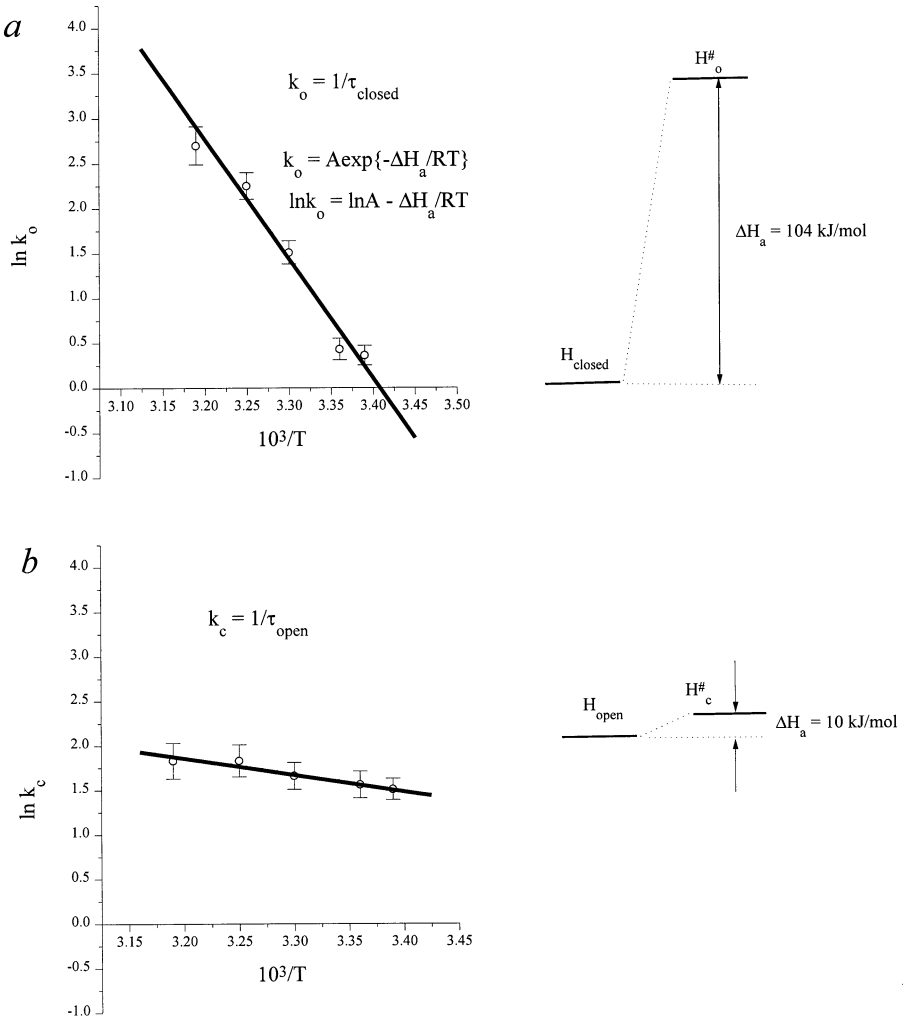

Fig. 2. Arrhenius plots for the rate constants of the single channel opening and closing. Rate constants for each single channel record were calculated as $k_o = \tau_c^{-1}$ and $k_c = \tau_o^{-1}$. From five to seven different single channel records were used to calculate mean value and standard deviation for time constants at each temperature. Activation enthalpy ΔH is defined as $k = A \exp(-\Delta H/RT)$. The best linear fits of the graph of ln k versus $1/T$ reveal the activation enthalpy for the channel opening (a) to be 104 kJ/mol and 10 kJ/mol for the channel closing (b).

pH = 7.2. Protein kinase A catalytic subunit (70 units/ml) and MgATP (2 mM) were added to the *cis* side only. Under these conditions any cationic channels present in the membrane vesicles are invisible and CFTR chloride channels appeared only after fusion of vesicles with an inside out orientation. Conductance as a function of Cl^- concentration and temperature as well as anion selectivity of the CFTR ion channel were tested in control experiments and showed good agreement with published data [11,17,18].

2.3. Materials

Phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine were from Avanti Polar Lipids. PKA catalytic subunit was from Promega. All other chemicals were from Sigma.

3. Results

Wild-type CFTR single channels were recorded after fusion of membrane vesicles with a preformed lipid bilayer. The electrophysiological properties of these channels closely corresponded to the properties of the wild-type CFTR ion channels measured before in excised patch experiments and reconstituted in lipid bilayers [11,16–18].

Two min single channel records at four different temperatures are shown in Fig. 1 as examples of the original records used to calculate time constants. All points histograms placed to the left of the records were used to calculate the probability for the channel to be open (P_o). Time constants of the CFTR ion channel gating mechanism were extracted from the dwell-time histograms shown to the right of the records. The closed state dwell-time histograms show two different closed time constants – fast and slow. The value of the fast time constant appeared out of our bandwidth and we have recorded only the tail of its distribution. Moreover, at +35°C and +40°C it was not detectable at all. However, no increased open time correlated to this loss of fast closing. Therefore the brief

Table 1

	τ_c (ms)	τ_o (ms)	$\tau_o/(\tau_o+\tau_c)$	P_o
23°C	700 ± 100 (4)	220 ± 30 (4)	0.24	0.23
25°C	650 ± 100 (6)	210 ± 30 (6)	0.23	0.25
30°C	220 ± 30 (5)	190 ± 20 (5)	0.46	0.45
35°C	105 ± 25 (3)	160 ± 20 (3)	0.60	0.60
40°C	80 ± 15 (3)	180 ± 30 (3)	0.69	0.71

closed state must have become non-stable and collapsed to the long lasting closed state at these temperatures. Nevertheless, from the changes observed at the lower temperatures the fast time constant is much less temperature sensitive than the slow one. With temperature elevation from +25°C to +30°C the fast time constant decreased less than 1.2 times while the slow time constant decreased nearly 3 times. The open state dwell-time histograms reveal only one time constant and it appeared to be only slightly temperature dependent. The time constants and the P_o values are summarized in Table 1 as mean values from three to six different experiments. Rate constants were defined as follow: $k_o = 1/\tau_c$ and $k_c = 1/\tau_o$, where τ_c is the characteristic closed time and τ_o is the characteristic open time.

The most typical way to investigate the effect of temperature on reaction rate is to represent experimental data as an Arrhenius plot on the coordinates, ln k versus $1/T$, where k is a rate constant and T is temperature on the Kelvin scale. For all thermal-driven reactions this graph should be linear [19,20]. The negative slope of the graph represents the activation energy of the reaction E_a from the function $k = A\exp(-E_a/RT)$. Deviations from linearity should indicate a change in the heat capacitance or departure from thermal equilibrium [20,21,24]. In Fig. 2 the temperature dependence of the rate constants for the opening and the closing of the CFTR ion channel is shown in such a plot. The experimental points were well fitted by a linear function. The agreement of the experimental data with the Arrhenius equation is a reflection of an equilibrium behavior of the quantity $k(T)$ which is governed only by thermal processes [19]. Hence, we can conclude that CFTR ion channel gating or at least its rate-determining step does not require energy other than thermal energy, to be activated. From these graphs (Fig. 2a, b) activation energies for the channel opening and closing may be extracted as the slopes of the best fit linear functions. Numerical values of approximately 104 kJ/mol were obtained for the activation energy of CFTR channel opening and 10 kJ/mol for the activation energy of channel closing. For comparison, the energy of ATP hydrolysis is ~35 kJ/mol [22] and the typical activation energy for diffusion in water is ~10 kJ/mol [23]. The activation energy for channel opening appeared much larger than both of these values. Further, to prove not only that the rate limiting steps but that in fact all of the steps in the gating mechanism are thermal-driven reversible processes, we compared P_o values determined directly from the all points histograms as a ratio of area under the peak of open state current to the total area under the peaks (Fig. 1) with P_o values calculated from the time constants as $\tau_o/(\tau_o+\tau_c)$. The values determined in such different ways will only agree for a reversible thermal-driven process. As shown in Table 1, indeed they agree extremely well, indicating that no additional input of free energy is required for the gating. In order to understand the large value of activation energy for channel opening in view of the lack of a need for additional free energy input we can compare that value with the total enthalpy difference between open and closed state. For a reversible thermal-driven process, the equilibrium constant K_{eq} is:

$$K_{eq} = k_o/k_c = \exp(-\Delta G/RT),$$

where ΔG is the Gibbs free energy difference between the closed and open channel configuration. From thermodynamics we have:

$$\Delta G = \Delta H - T\Delta S.$$

This relates the Gibbs free energy difference to the differences in enthalpy, ΔH and entropy, ΔS. In crude terms, ΔH is related to the formation, breaking or distortion of bonds, while ΔS is related to the change in order or randomness in the system.

One means of estimating the relative contributions of ΔH and ΔS to the change in the Gibbs free energy is to employ the Van't Hoff equation:

$$\ln K_{eq} = -\Delta H/RT + \Delta S/R.$$

This equation suggests that a linear plot of ln K_{eq} versus $1/T$ should yield $\Delta H/R$ from the slope of the best linear fit of the experimental points and the $\Delta S/R$ value from the intercept. The temperature dependence of the equilibrium constant K_{eq} is shown in Fig. 3. The experimental points reveal good linear dependence. The slope of the best linear fit gives a value of $\Delta H = -90 \pm 5$ kJ/mol and the intercept gives a value of $\Delta S = 295 \pm 15$ J/mol*K. For example, at +25°C (298 K) the term $T\Delta S = 87 \pm 5$ kJ/mol or $\Delta H \approx T\Delta S$ and $\Delta G_{25} = -3.0 \pm 0.1$ kJ/mol or just 1.2 RT. We find that the activation energy for channel opening, $H_o^{\ddagger}$ is similar to ΔH. Taken together this means that the configuration of the ion channel in the transition state structure for opening is the same or very close to the open channel configuration and practically all excess energy is used to diminish the entropy of the open state.

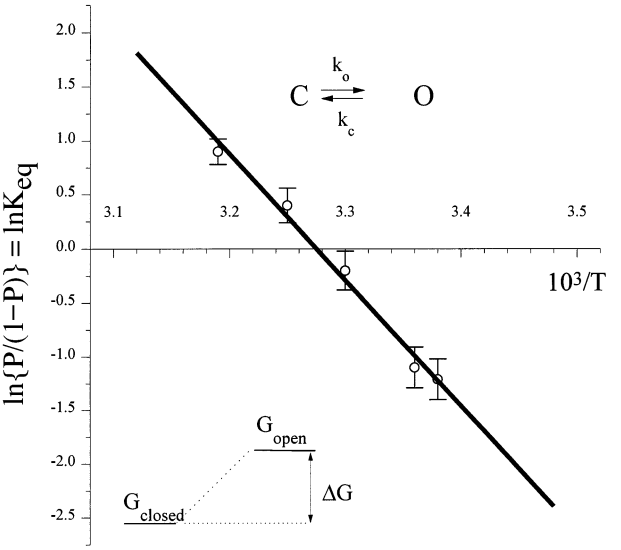

Fig. 3. Temperature dependence of the equilibrium constant. Equilibrium constant K_{eq} was calculated as a function of P_o at each temperature. If $k_o = \tau_c^{-1}$ and $k_c = \tau_o^{-1}$ then $K_{eq} = P/(1-P)$ and $\ln K_{eq} = \ln\{P/(1-P)\} = -\Delta G/RT = -\Delta H/RT + \Delta S/R$. Values of $\Delta H = 89 \pm 5$ kJ/mol and $\Delta S = -294 \pm 16$ J/mol*K were found from the best linear fit. $\Delta G = \Delta H - T\Delta S \ll \Delta H$ which means that all energy excess is used to diminish the entropy of the open state.

4. Discussion

The Arrhenius plots provided the activation energies for the

gating process. Of course, the physical meaning of the activation energy for processes in aqueous solution involving protein macromolecules is not nearly so clear as for chemical reaction in the gas phase [20,29]. The validity of the basic assumptions established for Eyring rate theory is questionable for enzyme catalyzed processes [24,25]. For this reason we used Arrhenius plots primarily to verify the thermal-driven nature of the rate limiting step rather than to estimate the contribution of activation entropy and free energy in the total activation enthalpy. Nevertheless it is possible to estimate and compare total activation enthalpies for the direct and reverse processes in the ion channel gating. From our numerical estimation the activation enthalpy for the channel opening ($H^{\ddagger}_{O}$) is much greater than that for the channel closing ($H^{\ddagger}_{C}$), $H^{\ddagger}_{O} \gg H^{\ddagger}_{C}$. On the other hand we have $H^{\ddagger}_{O} - H^{\ddagger}_{C} = \Delta H \approx T\Delta S$. Combining these we can write $H^{\ddagger}_{O} \approx \Delta H \approx T\Delta S$ which means that the activation entropy for the opening is of the same order as the entropy difference between the closed and open state or, in other words, the CFTR molecule(s) interconversion from the transition state for the opening to the open channel structure involves only a small reorganization of molecular structure.

From the numerical estimation of the $T\Delta S$ value we can conclude that a large entropy loss is required to open the channel. The only reagent needed to open the prephosphorylated CFTR ion channel is MgATP. We find no evidence that the free energy of ATP hydrolysis is needed for channel gating. Alternatively, we postulate that formation of the CFTR-MgATP complex induces formation of the open channel structure. This structural reorganization is the rate limiting step in the process of the channel opening. The energy of the enzyme-substrate interaction is used to 'pay for' the large entropy loss in channel opening.

The value of the activation enthalpy for channel closing was found to be approximately that of diffusion processes in water. Hence, the major component in the activation enthalpy of the closing is that for diffusion. We speculate that closing of the channel is induced by the increased diffusional motility of CFTR molecule segments after hydrolysis of the nucleotide triphosphate. In any case with an activation energy required to close the channel less than 10% of the activation energy for opening there is no chance of an entropy decrease as there was for opening. Therefore, no additional ATP binding followed by substantial reorganization in the CFTR structure is involved in the channel closing. If it were, then a strong temperature dependence of the closing rate constant would be observed and it was not.

This interpretation of our findings draws an analogy between the structure-fitting step in the Koshland's 'induced fit' model for enzyme catalysis [26–28] and the gating mechanism in CFTR ion channel function. The same CFTR molecule serves both processes i.e. ATP hydrolysis and ion channel gating. They also both require structural reorganization by thermal-driven processes. The apparent contradiction between a requirement for ATP hydrolysis in CFTR ion channel gating and the thermal-driven nature of the gating process may be resolved if we suppose structural coupling between the transition state structure of the CFTR-ATP-Mg enzyme-substrate complex and open channel structure instead of energetic coupling between them. If the transition state structure and the open channel structure coexist, then ATP hydrolysis is needed to make the gating process reversible. In this simplest model, ATP hydrolysis is the price of the reversibility of channel opening.

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