Nanoscale

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Nanoscale*, 2022, **14**, 13315

Single-molecule evidence for a chemical ratchet in binding between the *cam* repressor and its operator†

Hiroyuki Kabata, ‡[a] Hironori Aramaki §[b] and Nobuo Shimamoto *[a]

The affinity for regulator–operator binding on DNA sometimes depends on the length of the DNA harboring the operator, which is known as the antenna effect. One-dimensional diffusion along DNA has been suggested to be the cause, but this may contradict the binding affinity independent of the reaction pathways, which is derived from the detailed balance of the reaction at equilibrium. Recently, the chemical ratchet was proposed to solve this contradiction by suggesting a stationary state containing microscopic non-equilibrium. In a single-molecule observation, *P. putida* CamR molecules associate with their operator *via* one-dimensional diffusion along the DNA, while they mostly dissociated from the operator without the diffusion. Consistently, the observed overall association rate was dependent on the DNA length, while the overall dissociation rate was not, leading to an antenna effect. *E. coli* RNA polymerase did not show this behavior, and thus it is a specific property of a protein. The bipartite interaction domains containing the helix-turn-helix motif are speculated to be one of the possible causes. The biological significance of the chemical ratchet and a model for its microscopic mechanism are also discussed.

Received 23rd June 2022,
Accepted 23rd August 2022

DOI: 10.1039/d2nr03454a

rsc.li/nanoscale

Introduction

Many DNA-binding proteins bind to a limited number of DNA sites with high affinity, namely their specific sites. They also bind to other DNA sites known as nonspecific sites with much lower affinity. The complexes thus formed are named specific and nonspecific complexes, respectively. An operator is thus a specific site of its regulator protein. Nonspecific sites compete with specific sites for protein binding, while they provide a one-dimensional space for some proteins to diffuse along DNA,[1] which has been established by photo-cross linking,[2] processivity of enzyme action,[3] and single-molecule assays.[4]

In determining the affinity of the *E. coli* repressor TrpR for its operator *trpO* by a quantitative footprinting technique, we found that the longer the DNA harboring *trpO*, the larger the affinity for the *trpO* site. This effect has been termed the "antenna effect",[5,6] and TrpR has showed the largest effect so far of 10 000-fold.[7] Its length dependence is quantitatively explained by a composite equation involving the diffusion equation and rate equation, which claims that a longer DNA segment provides an increasing number of nonspecific sites for a protein to bind to and accelerates the formation of a specific complex with rapid one-dimensional diffusion, but the dissociation of the specific complex is less dependent on the length.[7] The involvement of one-dimensional diffusion was evidenced to some degree by the disappearance of the antenna effect by blocking the one-dimensional diffusion by the biotin–avidin joint between *trpO* and nonspecific sites *in vitro*. Moreover, the effect also disappeared *in vivo* by blocking the diffusion by the introduction of a LexA site near *trpO*.[8]

Our interpretation of the antenna effect of TrpR–*trpO* binding was challenged as a violation of the detailed balance of the rate equation. However, this criticism mistakenly supposes that detailed balance is a general truth, but this balance is actually an assumption required to describe a reaction with a single set of rate equations.[9] If there is a degree(s) of freedom with a timescale close to or slower than that of the reaction, a conformational change, for example, detailed balance, no longer holds.[7–11] In this case, the reaction is described with two or more sets of rate equations switching

[a]*National Institute of Genetics, and Department of Genetics, School of Life Science, The Graduate University for Advanced Studies, SOKENDAI, 1111 Yata, Mishima, Shizuoka, 411-8540, Japan. E-mail: nshima@nig.ac.jp*
[b]*Department of Molecular and Life Science, Faculty of Pharmacy, Daiichi University of Pharmacy, 22-1 Tamagawa-cho, Minami-ku, Fukuoka, 815-8511, Japan*

†Electronic supplementary information (ESI) available: 1. definition of chemical ratchet, 2. Additional specific sites, 3. energetic and geometric terms composing a rate constant. See DOI: https://doi.org/10.1039/d2nr03454a

‡Present address: Headquarters for Innovative Society-Academia Cooperation, University of Fukui, 3-9-1 Bunkyo, Fukui, 910-8507, Japan.
§Present address: Sankyu Drug Co., Ltd, 3-1-13 Kurogawa-Nishi, Moji-ku, Kitakyushu, 801-0825, Japan.

alternatively for each reactant molecule according to the degree(s) of freedom. We named this mechanism the chemical ratchet because it generates a circulating stationary flow among the free components, nonspecific complex and TrpR–*trpO* specific complex in this case.[7,10,11] Namely, the chemical ratchet is a mechanism where the detailed balance of a reaction is invalidated by an additional degree(s) of freedom with a timescale close to or slower than that of the relevant reaction (more details in ESI section 1†).

The evidence so far obtained is still insufficient to determine if TrpR–*trpO* binding is a chemical ratchet. If a deviation from detailed balance is observed as the behavior of a single reactant molecule, the corresponding macroscopic rate constants should show an antenna effect, providing positive evidence for the chemical ratchet. Unfortunately, TrpR–*trpO* binding has a timescale too rapid to determine the rate constants with a gel-shift assay, and we must employ a different protein and operator, *P. putida* CamR and *camO*, as experimental materials. *P. putida* CamR and *E. coli* TrpR belong to the same family of bacterial repressors binding to a palindromic sequence as homodimeric molecules with a helix-turn-helix motif.

According to the results of a single-molecule one-dimensional diffusion assay, the specific complex is preferentially formed *via* one-dimensional diffusion from the nonspecific complex, while it is preferentially dissociated without one-dimensional diffusion, driving a circulating flow. The measured overall rate constants and affinities on different DNA lengths showed a significant antenna effect. Therefore, CamR–*camO* binding forms a chemical ratchet.

Synopsis

• In a single-molecule assay with immobilized DNA, a CamR molecule prefers to bind to the *camO* site *via* one-dimensional diffusion along the DNA, while it tends to leave from the site to the bulk without the diffusion.

• This asymmetry in the binding reaction provides microscopic and direct evidence for the chemical ratchet, which has been proposed to explain the antenna effect of a protein belonging to the same structural group, *E. coli* TrpR. In a gel-shift assay, CamR also showed an antenna effect of more than 100-fold.

• In a binding reaction, a chemical ratchet occurs when a conformational change affecting the binding equilibrium has a timescale comparable to that of the binding reaction. The mechanism is microscopically described as switching two or more sets of rate equations. Such a reaction system becomes indifferent to detailed balance in its stationary state because it is microscopically non-equilibrated in these timescales. Therefore, in the case of CamR–*camO* binding, the major association is accelerated by one-dimensional diffusion dependent on DNA length, while the major dissociation is independent of the length, making the affinity dependent on the length, *i.e.* the antenna effect.

• The observed single-molecule behavior is specific to the DNA-binding protein. In the case of CamR and TrpR, the DNA bending in the operator complex can be an example of the conformational change essential to the chemical ratchet. Its indifference to detailed balance and its consistency with regards to switching are discussed for the complex structure.

• The biological significance of the chemical ratchet is discussed, and a novel cross-talk between two regulatory mechanisms, which have been ignored, is proposed.

Results

Behavior of *CamR* molecules in a single-molecule assay

CamR is a repressor regulating the expression from the *cam* operon responsible for the catabolism of D-camphor.[12] It belongs to the same family of bacterial repressors as *E. coli* TrpR. The 48 kDa CamR is composed of two identical subunits, and their helix-turn-helix motifs recognize the single 26 bp palindromic sequence of *camO*. CamR was prepared from *P. putida* and fluorescently labeled as described previously.[13,14] We examined the dynamic behavior of CamR molecules during the binding reaction with DNA harboring the *camO* site. The λ^{gt11} DNA molecules harboring *camO* were biotinylated at their 5′-ends and then dielectrophoresed between aluminum electrodes that were formed on an avidin-coated glass surface. The double-stranded DNA chains tend to form DNA belts, which are bundles of parallel DNA with its end fixed near an electrode edge and the other fixed on the glass surface *via* avidin during dielectrophoresis. The fixed DNA was kept extended and arrayed in parallel in the belts after washing in the absence of an electric field (Fig. 1A and B). Similar DNA belts were successfully used in the earliest single-molecule study for one-dimensional diffusion along DNA.[4]

Fluorescently visualized CamR molecules were injected at an angle to the DNA belt. If a CamR molecule diffuses one-dimensionally along the DNA, the bulk flow will drive it along the DNA at an angle to the flow, and its diffusion will be observed as a linear motion parallel to the fixed DNA molecules. As evidenced below, the direction of the bulk flow was not disturbed by the fixed DNA, which has also been shown before.[4]

The movements of the visualized CamR molecules were microscopically observed and displayed in the stroboscopic pictures as bright lines or spots, and the small and rapid Brownian motion is reflected as small fluctuations of the lines with gradation or blurring of the spots. The traces were classified into five modes by the criteria experimentally suggested in a previous study.[4] Since a DNA molecule was fixed at only both its ends, the unfixed part fluctuated within 2 μm in response to bulk flow. The continuous observation of CamR molecules was limited to *ca.* 10 s because of photo-bleaching of the fluorescent label. Linear motions longer than 3 μm were classified as "one-dimensional diffusion" or "simple drift" depending on

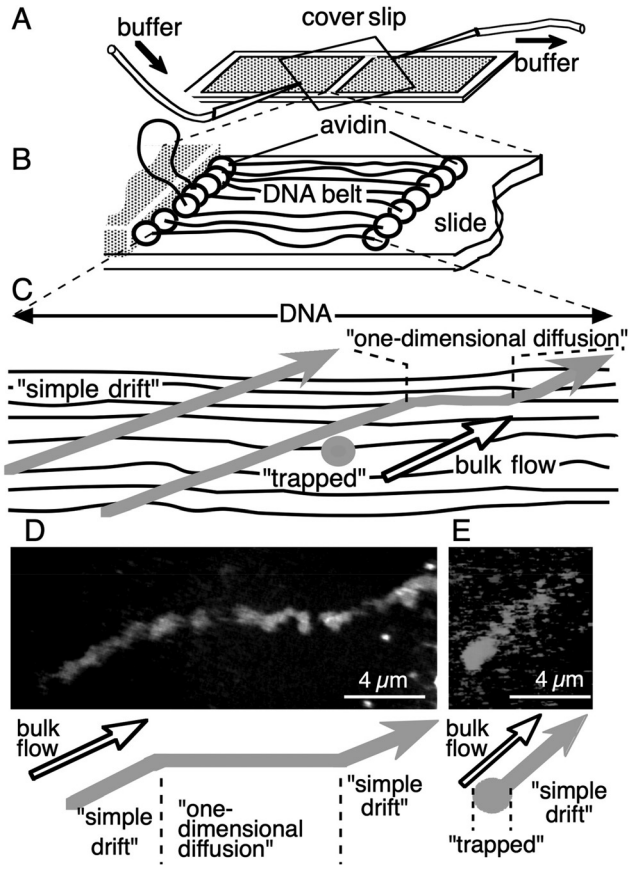

Fig. 1 Single-molecule behavior of CamR in a DNA belt. (A) Illustration of the one-dimensional diffusion assay with DNA belts that are formed between two aluminum electrodes (see Materials and methods). A laminar flow of the buffer was applied with a push–pull pump. (B) Illustration of a DNA belt with DNA fixed at both ends through end-attached avidin molecules. One looped DNA molecule is also illustrated at an electrode edge. (C) With the bulk flow (open arrow), the traces (gray arrows) are schematically shown for the modes of "simple drift", "one-dimensional diffusion", and "trapped". (D) A stroboscopic trace, a superimposed video frame, for 5 s of a CamR molecule that successively showed "simple drift", "one-dimensional diffusion", and "simple drift". (E) A stroboscopic trace of a "trapped" molecule that dissociated with no detectable one-dimensional diffusion. The preceding "one-dimensional diffusion" trace has been omitted from the photo. This is the major behavior associated with dissociation.

whether their directions were parallel to the extended DNA or to the bulk flow, respectively, within ± 10° (Fig. 1C and D). Blurring of spots due to the vertical movements of molecules, which occurred only near the electrode edges (Fig. 1B), was termed as "jumping". This is likely to be the one-dimensional diffusion along a looped DNA molecule, both ends of which were occasionally fixed at one electrode edge. A molecule that stayed within a circle of 2 μm diameter for more than 10 s was classified as "trapped" (Fig. 1C and D).

The direction of bulk flow was monitored *via* the movements of CamR molecules outside the DNA belt. The stroboscopic traces were classified only when the measured direction of bulk flow was maintained prior to and after the CamR molecules passed through a DNA belt. In a field of view of 50 μm^2, there was an attempt to maintain the direction of flow at an angle to the DNA belt by adjusting the pump outlet and the drain of the CamR solution at the diagonal corners of the square cover glass.

However, an unexpected distortion of the glass occasionally made the direction of flow parallel to the DNA belt, preventing a distinction between "one-dimensional diffusion" and "simple drift". Such cases were classified as "unidentified". When a trace contained two or more modes, it was classified according to the hierarchy of "trapped", "jumping", "one-dimensional diffusion", "simple drift", and "unidentified", as shown in Fig. 2, where a trace is uniquely classified in a single mode to make the total fractions to be 100%. For example, the trace shown in Fig. 1D displayed both simple drift and one-dimensional diffusion, and thus it was counted as "one-dimensional diffusion".

The fractions of modes in the standard assay condition (see Materials and methods) are shown as the top bar in Fig. 2, and more than half of the traces are classified in one of the DNA-interacting modes of "trapped", "jumping" or "one-dimensional diffusion". In a control experiment, where the DNA binding domain of CamR was blocked with a two-fold molar excess of 32 bp *camO* DNA, the frequencies of these three modes decreased dramatically, as expected (the second top bar). This result refutes the possibility of misinterpreting the observed "one-dimensional diffusion" mode as "simple drift" due to a rectified flow parallel to the DNA. Therefore, DNA fixed in parallel is essentially transparent for bulk flow at the experimental density of fixing. The absence of such a hydrodynamic artifact is also confirmed by examining the traces of fluorescently labeled DNA molecules in the absence of CamR (the fourth bar).

Next, we added the inducer of the operon, D-camphor, in the single-molecule assay (the third bar). Only the mode of "trapped" was affected by the inducer, and therefore, the inducer distinguishes a specific complex from a one-dimensional diffusion complex. This result agrees with a report that the *lac* repressor forms inducer-resistant complexes at nonspecific sites.[15]

The DNA binding site of CamR includes two identical domains, each of which has a single helix-turn-helix motif. We tested the possibility that the binding site can simultaneously interact with two DNA molecules during one-dimensional diffusion. Instead of CamR, unfixed 32 bp *camO* DNA was fluorescently visualized. The DNA was injected (the second bottom bar in Fig. 2), and the DNA that had been mixed with an 8-fold excess of unlabeled CamR was injected (the bottom bar). If the observed one-dimensional diffusion contains the putative bridging of two DNA molecules by CamR, there would be a significant increase in the one-dimensional diffusion mode in the bottom bar, but this was not observed. Therefore, all three DNA-interaction modes require the whole DNA-binding site on a CamR homodimeric molecule, at least in this condition.

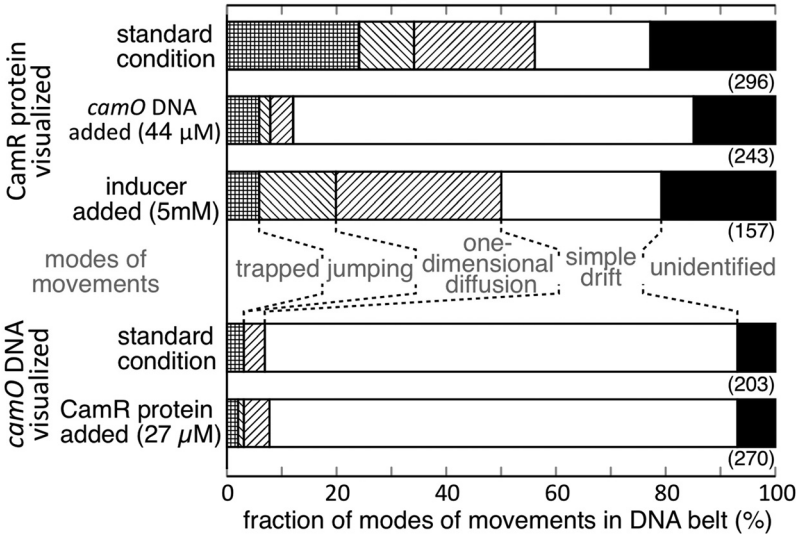

Fig. 2 Fractions of the observed modes of single molecules of CamR and *camO* DNA. The traces containing two or more modes are classified according to the hierarchy shown in the line of "modes of movements" from left to right. The total number of the classified traces is shown in parentheses below the right end of each bar. In the experiments with added components, the injections were performed after at least 40 min incubation after the additions.

Asymmetric use in pathways of association to *camO* and dissociation from it

Among the 71 observed trapping events in the standard condition (24% of analyzed traces in the top bar in Fig. 2), three-fourths occurred at the position that agrees with the *camO* on the λ^{gt11} DNA within the resolution of microscopic observation (see ESI section 2†). The remainder was observed at two other sites: near an end and at a position 4 μm from an end. These are speculated to be two sites homologous to *camO* on the DNA, one at the λ coordinate 1588 (0.5 μm from the "left" end) with 87% identity to the 15 bp central palindrome and the other at 12 477 (4.2 μm from the "right" end) with 80% identity. DNA fragments harboring these sites compete with DNA harboring *camO* in a gel-shift assay. Therefore, they are specific sites for CamR and "trapped" is attributable solely to the formation of specific complexes. In other words, most nonspecific complexes of CamR can fall into the category of one-dimensional diffusion complexes, showing the reality of the bipartite classification of DNA sites into specific and nonspecific.

Notably, all the 71 traces classified as "trapped" in the standard condition showed the "one-dimensional diffusion" mode prior to "trapped" events, as has also been observed for *E. coli* RNA polymerase.[4] However, "one-dimensional diffusion" was rarely observed in the dissociation from the specific sites. Among the 71 traces, 35 dissociated from the specific sites and 36 remained "trapped" (Table 1) during observation. Only one of the 35 dissociated had a detectable one-dimensional diffusion motion, and the remaining 34 dissociated showed no sign of "one-dimensional diffusion", as shown in the example in Fig. 1E. This significant bias was not observed for RNA polymerase.

Table 1 Asymmetric use of direct and indirect pathways of association and dissociation in the microscopic behavior of CamR single-molecules

	Association to "trapped"		Dissociation from "trapped"	
Binding pathways	Cases	(%)	Cases	(%)
Indirect pathway accompanied by one-dimensional diffusion or jumping	71	(100)	1	(3)
Direct pathway into bulk without one-dimensional diffusion or jumping	0	(0)	34	(97)
Total	71	(100)	35	(100)

These results mean that the one-dimensional diffusion of CamR occurs more frequently in the overall association than in the overall dissociation. This conclusion is not altered even if all the 36 molecules that remained "trapped" during observation ultimately slid out of their specific sites after our observation because at most 37 molecules should slide out from the sites among the 71 molecules that experienced "one-dimensional diffusion" into the "trapped" sites. This is consistent with the interpretation that CamR associates to *camO* mainly in the pathway mediated by one-dimensional diffusion, while it dissociates from the site mainly *via* the direct pathway without diffusion, which is predicted by the chemical ratchet mechanism as a deviation from the detailed balance in the binding of TrpR to its operator.[7,11]

This asymmetry was not detected for RNA polymerase, and its dissociation from the promoter was mostly accompanied by one-dimensional diffusion. We did not observe any distinct differences between CamR and RNA polymerase in the same one-dimensional diffusion assay except for the asymmetry

found for CamR. Their diffusion distances were likely to be comparable, although we did not quantify the distances. Therefore, the largest difference between them is the strong block of the diffusion from *camO* to its adjacent sites. This might suggest that the conformation of the CamR–*camO* complex has been changed before dissociation.

Antenna effect consistent with single-molecule evidence

When the single-molecule behavior of molecules is interpreted, there is generally a danger due to the artifacts caused by chemical modification, fixing, and several surface effects. The observed asymmetric behavior in association and dissociation must be macroscopically confirmed in solution. The binding between CamR and DNA fragments harboring the *camO* operator with lengths of 32, 136, and 400 bp was analyzed by three mobility-shift assays (Fig. 3A). The solutions in dissociation and association kinetic measurements were manually mixed on a piece of thermostated paraffin film, followed by the rapid loading of a sample in a gel slot.

The macroscopic counterpart of the asymmetry is the difference in the DNA-length dependence of the overall association rate constant and overall dissociation rate constant, as shown in Table 2. The existence of "one-dimensional diffusion" prior to "trapped" with no exception was consistent with the observed increase of two orders of magnitude of the association rate constant on increasing the DNA length from 32 bp to 410 bp. In contrast, the rare cases of "one-dimensional diffusion" following "trapped" were macroscopically reflected by the maintenance of the dissociation rate constant within two-fold for the same increase in the length. As a result of these different effects on the rate constants, their ratio, the affinity of CamR for *camO*, should experience an antenna effect of two orders of magnitude. In fact, a 110-fold antenna effect was observed in the direct and more accurate measurement, as shown in Table 2. Therefore, CamR with a single DNA binding site shows the antenna effect because of the biased use of one-dimensional diffusion in association and dissociation.

Since the antenna effect is named regardless of its mechanism, it is also caused by a conventional mechanism wherein a protein molecule has two DNA binding sites to form a complex with a DNA loop.[17–21] Since this looping costs energy because of the rigidity of double-stranded DNA, the longer the DNA, the larger the affinity. Thus, the looping mechanism is not possible for DNA much shorter than the persistence length as well as DNA long enough to be flexible. In fact, this mechanism has been denied for TrpR by an experiment using a biotin–avidin connection.[7] For these reasons, the antenna effect of *Eco*RI DNA methyltransferase seems to be caused by a chemical ratchet.[22]

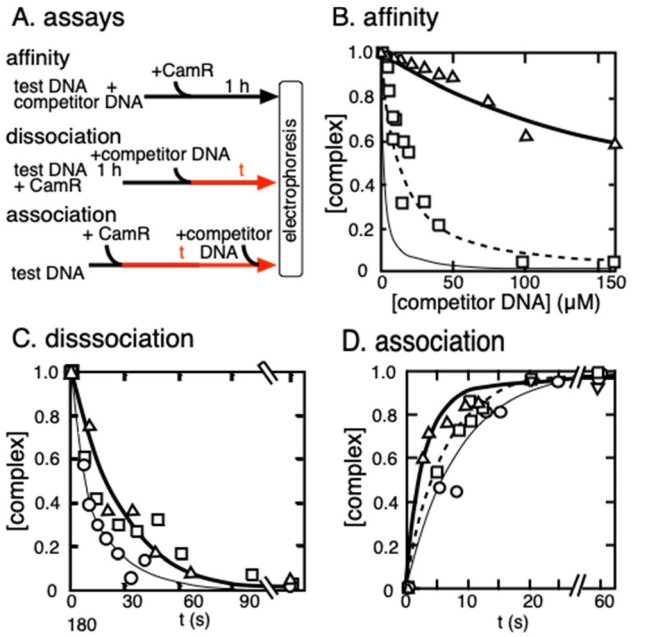

Fig. 3 The measurements of the affinity, dissociation rate constant, and association rate constant for CamR–*camO* binding by gel-shift assays. We normalized the amount of CamR complexed with a ^{32}P-labeled test DNA with respect to that with no competitor. The tested DNAs harbor the same 26 bp *camO* sequence at their centers with lengths of 32 bp (circles), 136 bp (squares), and 410 bp (triangles). The lines are least-square-fit curves and are drawn in thin solid (32 bp), dotted (136 bp), and thick solid lines (410 bp). (A) Diagram of the assays. The line in red represents the measuring time point, t, and that a tested DNA was ^{32}P-labeled at its 5′-end and was challenged by the competitor DNA to form the complex. (B) Complex formation after incubation for 1 h. The labeled test DNA was premixed with the competitor (unlabeled 32 bp DNA), and CamR was then added, followed by incubation. For the 32 bp DNA, the experiment was essentially radioactive dilution, and so the dilution curve is presented with no data points (thin solid line). (C) Time courses of the dissociation of CamR–test DNA complexes. An excess amount of the competitor was added at time zero. At each time point, *t*, an aliquot was withdrawn with a pipette tip containing 1/5 volume of 50% glycerol and rapidly mixed with blowing/sucking on a piece of paraffin film just before loading on the gel to which an electric field was applied. The data at time zero were obtained by loading the mixture of the labeled DNA and CamR. The best-fit curves for the 136 bp and 410 bp DNAs overlap with each other. (D) Time courses of association. One of the labeled test DNA was mixed with a CamR solution at $t = 0$. The competitor DNA in 20% glycerol was added at time t to prevent further association.

Discussion

The common feature of the one-dimensional diffusion of CamR and RNA polymerase involves the apparent "friction" between the protein and DNA. For both CamR (in this study) and RNA polymerase,[4] the speed of a protein molecule dragged by the flow was between 30% and 70% of the parallel component of the flow, corresponding to a height of 0.4–1.2k_BT, with the absolute temperature T with the Boltzmann constant k_B. It is close to the average energy of thermal fluctuations and realizes efficient one-dimensional diffusion, an essential part of the antenna effect. The barrier between the specific site and an adjacent nonspecific site should be similarly low; otherwise, one-dimensional diffusion would not accelerate the formation of the CamR–*camO* complex.

Table 2 Antenna effect and rate constants of CamR binding to *camO*

DNA length[a] (bp)	Antenna effects: K_d observed[b] (nM)	Antenna effects: Relative affinities	Rate constants: Association rate constant (10^4 M^{-1} s^{-1})	Rate constants: Dissociation rate constant (s^{-1})
32	570 ± 60[c]	(1)	9.5 ± 6	0.054 ± 0.008
136	80 ± 10	7.1	40 ± 30	0.032 ± 0.006
410	5.0 ± 0.6	110	710 ± 300	0.036 ± 0.004

[a] DNA harbors the 26 bp *camO* segment at the stem of the hairpin or at the centers of the fragments. [b] K_d calculated from the competition with 32 bp *camO* DNA. [c] K_d of hairpin DNA determined by fluorescence titration.[16]

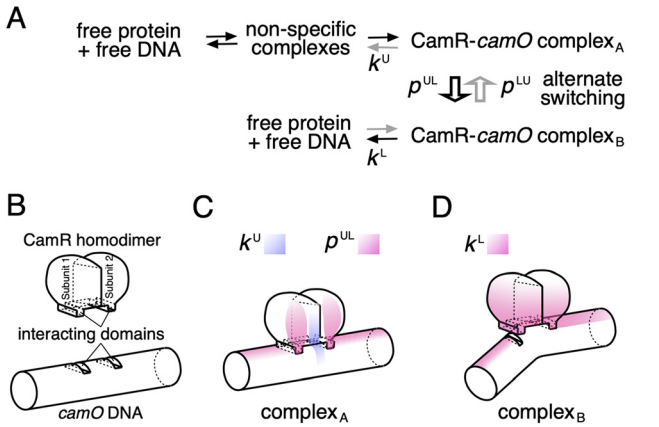

Fig. 4 A possible model for the chemical ratchet of CamR–*camO* binding. (A) The minimal mechanism of CamR–*camO* binding, which is essentially the same as the binding of TrpR–*trpO*.[8] According to the conformational change of the CamR–*camO* complex, the two sets of rate equations shown in the upper and lower lines alternately switch to the other (open arrows) with the possibilities per unit time being p^{UL} and p^{LU}, respectively. If $complex_B$ has too short a lifetime for k^L to be defined, the p^{UL} and k^L steps are essentially considered to be an alternative dissociation with a rate constant of $p^{UL}k^L/(p^{LU} + k^L)$. The steps attenuated in a chemical ratchet are shown with dimmed arrows. (B) Symbolic illustration of a bacterial repressor and its operator. (C) Symbolic illustration of $complex_A$. The distribution of the area for effective collision for the k^U process is shown in blue gradation, and that for p^{UL} is shown in red gradation. (D) Symbolic illustration of $complex_B$. The distribution of the area for effective collision for the k^L process is shown in red gradation.

Because of the structural similarity and large antenna effects, it is not unnatural to speculate that CamR–*camO* binding and TrpR–*trpO* binding share a common molecular mechanism. We showed positive evidence for the former being a chemical ratchet as the behavior of molecules is indifferent to detailed balance, which is an essential property of the latter (ESI Table S1†). As long as the binding is a chemical ratchet, there should be a degree(s) of freedom whose timescale is close to that of the binding reaction, but this has not been identified in any experiments. To establish the reality of the chemical ratchet, we must present a consistent example on the assumption that the freedom(s) is the conformational change of the CamR–*camO* complex (Fig. 4).

In a microscopic view, the complex is either in the conformation of the stable $complex_A$ or unstable $complex_B$. The binding reactions are described with their specific rate equations; the upper one for $complex_A$ and the lower for $complex_B$. These two rate equations are switched in time.[7,11] It should be noted that $complex_B$ could have a lifetime too short for its fate to be describable with a rate constant k^L. In that case, what is switched is the dissociation rate constant between the values of k^U and the one corresponding to $p^{UL}k^L/(p^{LU} + k^L)$. In a dominant chemical ratchet like CamR–*camO* binding, the major pathways are different in association and dissociation, as observed in the experiment. Thus, $p^{UL}k^L/(p^{LU} + k^L)$ should be larger than k^U, regardless of the lifetime of $complex_B$.

The dissociation processes denoted by the rate constants k^U and k^L, and the conformational change denoted by those of p^{UL} and p^{LU}, can be considered to be caused by collisions with the solvent molecules. Since the movements of small solvent molecules are thermally equilibrated in the timescale of these relevant processes of the complexes, the kinetic constant is the conversion efficiency per unit time from the collisions in the process that are summed over all the possible energies and orientations of the collisions. As described in ESI section 3,† the efficiency is described as a product of two terms. One is the potential difference between the transition and the ground states of the process, exp(−Δpotential), if Δpotential is standardized with k_BT. The other is the geometric factor determined by the structure of the reactant, $complex_A$ or $complex_B$. Note that the potentials are purely determined by the microscopic structure of the macromolecule, and are different from the macroscopic thermodynamic free energy containing entropy.

What is dominant in the geometric factor is the degree of exposure of the reactant molecule to the collisions of solvent molecules. In panels B–D in Fig. 4, we schematically illustrate $complex_A$ and $complex_B$ as well as CamR and a part of *camO* DNA. The bipartite interaction domain is drawn as two pairs of a square bar and a ditch, each pair of which is supposed to generate a stabilization energy of m (Fig. 4B). The process of $complex_A$ is either its dissociation (k^U) or its conformational change to $complex_B$ (p^{UL}). For the collisions to break both pairs of interaction domains at once to drive k^U, they have to be attacked simultaneously. Otherwise, a break preceding the other tends to lose the energy *via* a distortion of the soft protein body as dissipation into heat, driving p^{UL} instead of k^U (Fig. 4C). Therefore, the collisions driving k^U must hit the narrow area of equal distance from the pair, namely near the symmetric plane of the interaction domains (the blue gradation in Fig. 4C). Furthermore, the exposure against solvent for $complex_B$ is larger than that for $complex_A$ (Fig. 4C and D). Thus, the stepwise dissociation *via* $complex_B$ may have a larger geometric factor than the single-step one. The potential factors of the one-step dissociation and those of the two-step dissociation are almost equal (see ESI section 3† for details).

Therefore, one-step dissociation can be slower than the two-step one *via* complex$_B$, making the dissociation without one-dimensional diffusion the major dissociation pathway.

For the kinetic control of a reaction, a regulatory mechanism must have a timescale similar to that of the relevant reaction, *i.e.* timescale matching; otherwise, the regulation is not realized.[11] If so, one-dimensional diffusion of the protein cannot regulate gene expression because the binding has a typical timescale of seconds or less and that of gene expression is typically minutes to hours. However, the chemical ratchet converts the acceleration of the binding reaction into an enhancement of the time-independent affinity and realizes the control of gene regulation. Recently, fine tuning of the affinity for an operator has been suggested for homopolymeric and repetitive nucleotide sequences flanking the operator.[23–26] This is possibly explained as an antenna effect caused by one-dimensional diffusion, as such redundant sequences can induce diffusion over a short distance.[27] This avoidance of timescale matching is a biological significance of the chemical ratchet.

In the study of TrpR–*trpO* binding,[8] a binding site of LexA was introduced at a distal position where direct physical contact between LexA and TrpR was not possible. Notably, the binding of cellular LexA to the artificial site suppressed the repression by TrpR *in vivo*, and this was overcome by the overproduction of TrpR, suggesting that LexA binding reduces the affinity of TrpR for *trpO* by blocking the one-dimensional diffusion of TrpR. In other words, a new artificial cross-talk between LexA and TrpR was created by inserting a LexA binding site within the diffusion distance of TrpR from *trpO*. Thus, new cross-talks between two different regulator proteins can be predicted by examining the distance between their operators. This is the second and possibly more important biological significance of the chemical ratchet.

Conclusions

When two or more reactions share a timescale and are converged to the stationary state, unidirectional circulating flow among three or more reaction states can be generated from microscopic random thermal fluctuation. This mechanism, named the chemical ratchet, has been analyzed with a single-molecule one-dimensional diffusion assay of the CamR repressor and λDNA harboring its operator. In association with the operator, all CamR molecules were mediated by one-dimensional diffusion along DNA, while in dissociation from the operator site, in 34 out of 35 cases, they were not mediated by one-dimensional diffusion. This asymmetric use of one-dimensional diffusion leads to a unidirectional reaction flow of the dissociated state to nonspecific complex to operator complex. This result directly evidences the existence of a chemical ratchet. This should increase the rate of association with the DNA length while maintaining that of the dissociation constant, which was confirmed by kinetic analysis. As a possible model, the second reaction was speculated to be DNA bending at the operator site in the CamR–operator complex.

Materials and methods

Protein and DNA

We fluorescently labeled CamR with an F_{ab} fragment prepared from polyclonal antibody and conjugated with trimethylrhodamine-modified avidin. The purified CamR and its visualized derivative were 86% and 78% active, respectively, determined by co-sedimentation with λ^{gt11} DNA harboring the 26 bp *camO*. The 250 bp *Eco*RI fragment of the plasmid pHA37-1 containing the 26 bp *camO* site, GGCTCTATATCTGCGATATA CTGAGC,[13] was inserted into the *Eco*RI site of λ^{gt11} DNA (Agilent, Santa Clara). The DNA was packaged into bacteriophage particles, amplified, and purified to prepare λ^{gt11}::*camO* DNA. The 136 bp, 410 bp and 1444 bp DNA fragments containing the *camO* site at their centers were amplified using appropriate primers with pHA37-1 as the template. The biotinylation of DNA was performed as previously described.[4]

Single-molecule dynamics

The one-dimensional diffusion of CamR molecules was examined by the assay established for *E. coli* RNA polymerase[4] with small modifications. Belts of λ^{gt11}::*camO* DNA were formed on a glass microscope slide by extending and fixing the DNA (100 μg ml^{-1} in pure water) in parallel by dielectrophoresis at 1 MHz between aluminum electrodes with a gap of *ca.* 50 μm. The glass surface was treated with 3-glycidoxypropyltrimethoxysilane,[28] which stabilizes the fixing of the avidin attached at the DNA ends. The electric field was removed and the DNA belts were washed with the assay buffer containing 10 mM Tris-HCl (pH7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% (v/v) glycerol, and 0.5 mg ml^{-1} of alkaline-hydrolyzed casein, which minimizes the absorption of CamR onto the glass surface. It was assessed *via* confocal microscopy of the DNA belts stained with propidium iodide, affording an occupancy of less than 0.1% by DNA in the cross-section of the DNA belt. The images of DNA belts were consistent with DNA extended in parallel but 15% to 20% of them formed loops at the edges of the electrodes.

Fluorescently labeled CamR or DNA was injected with a hand-made pump into an observation chamber, which was supported on a plate thermostated at 25 °C (Fig. 1A). The injection and design of the chambers were such that the bulk flow and the fixed DNA intersected at about 45°, although this angle was sometimes changed due to various technical reasons. Since too slow a flow makes movements along the DNA indistinguishable from the Brownian motion of free protein molecules, the flow was adjusted to give a residence time between 0.5 s and 5 s in the belts.

Observations were made with a microscope (RFLBHS Olympus, Tokyo) equipped with a camera (C2400-08, Hamamatsu, Toyohashi), an observation chamber, a thermostated plate (KM-1, Kitazato, Shizuoka), a 40× objective lens

(UPlanAPO, Olympus, Tokyo), a mercury arc lamp (USH103D, Ushio, Tokyo), a neutral density filter of 25% transparency, and an upright housing (BX51, Olympus, Tokyo).[4]

Electrophoretic mobility-shift assays

All gel-shift assays were carried out in 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA at 25 °C. The test fragments were terminally labeled with ^{32}P and mixed with unlabeled competitor DNA, and the mixtures were incubated with CamR. Electrophoresis was performed in 4% or 6.5% polyacrylamide gels (15 cm × 15 cm) in the same buffer with circulation at 200–400 V for 1 h. The gels were dried, and their autoradiograms were recorded by a phosphoimager (BAS2000, Fuji Film, Tokyo). The solutions in dissociation and association kinetic measurements were manually mixed on a piece of thermostated paraffin film, followed by the rapid loading of a sample in a gel slot.

To confirm the absence of artifacts specific to mobility-shift assays, we carried out all conceivable control experiments: application of different voltages in the electrophoresis, identification of the one-to-one complex, mutual verification by measuring the radioactivity of each of the complex and free DNA bands, with a correction for the possible contributions of dissociation of the complex during electrophoresis.[4]

The dissociation rate constants of CamR from the three DNA fragments were measured by challenging the preformed CamR–*camO* complex with an excess amount of unlabeled competitor DNA (1444 bp plasmid DNA harboring *camO*). This method allows reliable measurement of rate constants smaller than 0.1 s^{-1}, and the values lay between 0.03 and 0.05 s^{-1} for these DNA fragments.

The association rate constants were measured by the addition of CamR to target DNA and a second addition of excess competitor DNA at various time points after mixing. The decrease in free target DNA was measured to calculate the complexed DNA at various time durations between two additions, as shown in Fig. 3A. This assay showed the poorest accuracy due to the two successive mixing steps, but was accurate enough to determine the first digit of the association rate constant.

Author contributions

H. Kabata performed all the bench works and analyzed the results, H. Aramaki supplied the materials and N. Shimamoto wrote the manuscript and is the member who proposed the concept of a chemical ratchet.

Conflicts of interest

The authors declare that they have no competing financial interests.

Acknowledgements

We thank Dr Jun-ichi Tomizawa for his critical help in constructing the logic and Ms. Harriet Sallach and Dr Pradip Bandyopadhyay for their helpful comments on the manuscript. We also thank the collaborators who proposed the concept of a chemical ratchet: Dr Mikito Toda, Dr Shigetishi Nara, Dr Tamiki Komatsuzaki, and Kiyoto Kamagata. Supported by JSPS KAKENHI (Grant-in-Aid for Scientific Research on Priority Areas) to NS (15030240) and to HK (15011206).

References

1 P. H. von Hippel and O. G. Berg, Facilitated target location in biological systems, *J. Biol. Chem.*, 1989, **264**, 675–678.
2 C. S. Park, F. Y. Wu and C. W. Wu, Molecular mechanism of promoter selection in gene transcription II. Kinetic evidence for promoter search by a one-dimensional diffusion of RNA polymerase molecule along the DNA template, *J. Biol. Chem.*, 1982, **257**, 6950–6956.
3 A. Jeltsch, J. Alves, H. Wolfes, G. Maass and A. Pingoud, Pausing of the restriction endonuclease *Eco*RI during linear diffusion on DNA, *Biochemistry*, 1994, **33**, 10215–10219.
4 H. Kabata, O. Kurosawa, I. Arai, M. Washizu, S. A. Margarson, R. E. Glass and N. Shimamoto, Visualization of single molecules of RNA polymerase sliding along DNA, *Science*, 1993, **262**, 1561–1563.
5 N. Shimamoto, One-dimensional diffusion of proteins along DNA: Its biological and chemical significance revealed by single-molecule measurements, *J. Biol. Chem.*, 1999, **274**, 15293–15296.
6 N. Shimamoto, Nanobiology of RNA polymerase: Biological consequence of inhomogeneity in reactant, *Chem. Rev.*, 2013, **113**, 8400–8422.
7 N. Shimamoto, M. Toda, S. Nara, T. Komatsuzaki, K. Kamagata, T. Kinebuchi and J.-I. Tomizawa, Dependence of DNA length on binding affinity between TrpR and trpO of DNA, *Sci. Rep.*, 2020, **10**, 15624.
8 T. Kinebuchi and N. Shimamoto, One-dimensional diffusion of TrpR along DNA enhances its affinity for the operator by chemical ratchet mechanism, *Sci. Rep.*, 2021, **11**, 4255.
9 N. Shimamoto, The limitation of the combination of transition-state theory and thermodynamics for reactions of proteins and nucleic acids, *Biomolecules*, 2022, **12**, 28.
10 N. G. van Kampen, *Stochastic Processes in Physics and Chemistry*, Elsevier, Amsterdam, The Netherlands, 3rd edn, 2007, pp. 114–171.
11 N. Shimamoto and M. Imashimizu, RNA polymerase and transcription mechanisms: The forefront of physicochemical studies of chemical reactions, *Biomolecules*, 2021, **11**, 32.
12 M. Fujita, H. Aramaki, T. Horiuchi and A. Amemura, Transcription of the cam operon and camR genes in

Pseudomonas putida PpG1, *J. Bacteriol.*, 1993, **175**, 6953–6958.

13 H. Aramaki, Y. Sagara, K. Takeuchi, H. Koga and T. Horiuchi, Nucleotide sequence of the gene encoding a repressor for the cytochrome P-450cam hydroxylase operon on the Pseudomonas putida CAM plasmid, *Biochimie*, 1994, **76**, 63–70.

14 H. Aramaki, Y. Sagara, H. Kabata, N. Shimamoto and T. Horiuchi, *In vitro* transcriptional analysis of the cytochrome P-450cam hydroxylase operon, *J. Bacteriol.*, 1995, **177**, 3120–3127.

15 A. Revzin and P. H. von Hippel, Direct measurement of association constants for the binding of Escherichia coli lac repressor to non-operator DNA, *Biochemistry*, 1977, **17**, 4769–4776.

16 H. Aramaki, H. Kabata, S. Takeda, H. Itou, H. Nakayama and N. Shimamoto, Formation of repressor-inducer-operator ternary complex: negative cooperativity of d-camphor binding to CamR, *Genes Cells*, 2011, **16**, 1200–1207.

17 A. Hochschild and M. Ptashne, Cooperative binding of lambda repressors to sites separated by integral turns of the DNA helix, *Cell*, 1986, **44**, 681–687.

18 H. P. Mueller-Storm, J. M. Sogo and W. Schaffner, An enhancer stimulates transcription in trans when attached to the promoter via a protein bridge, *Cell*, 1989, **59**, 767–777.

19 A. M. Khoury, H. J. Lee, M. Lillis and P. Lu, Lac repressor-operator interaction: DNA length dependence, *Biochim. Biophys. Acta*, 1990, **1087**, 55–60.

20 S. Oehler, E. R. Eismann, H. Kramer and B. Muller-Hill, The three operators of the lac operon cooperate in repression, *EMBO J.*, 1990, **9**, 973–979.

21 T. Harmer, M. Wu and R. Schleif, The role of rigidity in DNA looping-unlooping by AraC, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 427–431.

22 M. A. Surby and N. O. Reich, Facilitated diffusion of the *Eco*RI DNA methyltransferase is described by a novel mechanism, *Biochemistry*, 1996, **35**, 2209–2217.

23 A. Afek and D. B. Lukatsky, Nonspecific protein-DNA binding is widespread in the yeast genome, *Biophys. J.*, 2012, **102**, 1881–1888.

24 A. Afek and D. B. Lukatsky, Genome-wide organization of eukaryotic preinitiation complex is influenced by nonconsensus protein-DNA binding, *Biophys. J.*, 2013, **104**, 1107–1115.

25 A. Afek and D. B. Lukatsky, Positive and negative design for nonconsensus protein-DNA binding affinity in the vicinity of functional binding sites, *Biophys. J.*, 2013, **105**, 1653–1660.

26 A. Afek, J. L. Schipper, J. Horton, R. Gordân and D. B. Lukatsky, Protein-DNA binding in the absence of specific base-pair recognition, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 17140–17145.

27 J. M. Schurr, The one-dimensional diffusion coefficient of proteins absorbed on DNA hydrodynamic considerations, *Biophys. Chem.*, 1979, **9**, 413–414.

28 G. C. Paffenbarger, W. T. Sweeney and R. L. Bowen, Bonding porcelain to acrylic resin denture bases, *J. Am. Dent. Assoc.*, 1967, **74**, 1018–1023.