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Photoresponses of *Halobacterium salinarum* to Repetitive Pulse Stimuli

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ABSTRACT *Halobacterium salinarum* cells from 3-day-old cultures have been stimulated with different patterns of repetitive pulse stimuli. A short train of 0.6-s orange light pulses with a 4-s period resulted in reversal peaks of increasing intensity. The reverse occurred when blue light pulses were delivered as a finite train: with a 3-s period, the response declined in sequence from the first to the last pulse. To evaluate the response of the system under steady-state conditions of stimulation, continuous trains of pulses were also applied; whereas blue light always produced a sharply peaked response immediately after each pulse, orange pulses resulted in a declining peak of reversals that lasted until the subsequent pulse. An attempt to account for these results in terms of current excitation/adaptation models shows that additional mechanisms appear to be at work in this transduction chain.

INTRODUCTION

Halobacterium salinarum, a salt-pond archaeon, swims by rotating a flagellar bundle at the cell's pole; switching the flagellar motor from clockwise to counterclockwise or vice versa results in a reversal in swimming direction, thus allowing cells to sweep their environment. Light of different wavelengths and several chemicals can affect the reversal frequency and thus produce photo- and chemotaxis. Typically a step-up of blue or blue-green light elicits reversals, whereas a step-up of orange light induces smooth swimming (opposite responses occur when each of these lights is turned off). The photobehavior of *H. salinarum* is the output of a molecular transduction process, which has been extensively studied by spectroscopic, biochemical, and genetic approaches; this plentiful but still incomplete knowledge is the basis for understanding and discussing the swimming photobehavior of this microorganism.

Photoresponses in *H. salinarum* are basically attributed to excitation/adaptation processes. Excitation occurs because, after chromophore isomerization upon light absorption, conformational changes in the protein moieties of the photoreceptors can send signals to the flagellar motor switch. Orange and near-UV-blue responses are due to a single photoreceptor (sensory rhodopsin I or SR-I), whose ground state (SR_{587}), by absorbing orange light, undergoes a conformational change to S_{373}, which can be converted to S^{b}_{510} by blue light. Both S_{373} and S^{b}_{510} decay thermally to SR_{587}, with quite different time constants (Spudich and Bogomolni, 1983; Bogomolni and Spudich, 1987). Blue-green light is sensed through a second sensor (sensory rhodopsin II or SR-II), also displaying a photocycle with long-lived intermediates and eliciting photophobic responses (Spudich et al., 1986; Tomioka et al., 1986; Wolff et al., 1986; Takahashi et al., 1990). Clear evidence has been reported that S_{373} is the attractant signaling state in the photocycle of SR-I (Yan and Spudich, 1991).

However, to understand the behavior of *H. salinarum*, the photocycle is only a starting point. The behavioral output depends on the architecture of the transduction pathways (Marwan et al., 1995; Hoff et al., 1997). A basic phenomenon to be considered in trying to understand this behavior is adaptation. It is well known that photoresponses in *H. salinarum* are elicited by changes in light intensity, and this microorganism adapts to steady levels of light. Adaptation and excitation processes are the two sides of a single coin and work together to shape the response. The adaptation processes studied in the chemotactic behavior of *Escherichia coli* are due to the reversible methylation of the activated chemoreceptors (MCp, methyl-accepting chemotaxis proteins; for a recent review, see Eisenbach, 1995). In *E. coli*, the scheme of the transduction is the following: the activated receptor modulates the phosphorylation of CheA (an autophosphorylating histidine kinase); this, in turn, controls by phosphotransfer the activities of several Che proteins involved in chemotaxis. Two of the Che proteins are enzymes that methylate (CheR) or demethylate (CheB) the chemoreceptor, thus modulating its responsiveness.

Similar processes are present in *H. salinarum*, in which methylatable transducers strictly associated with sensory pigments have been found (HtrI for SR-I, HtrII for SR-II) (Yao and Spudich, 1992; Spudich and Spudich, 1993; Ferrando-May et al., 1993; Olson and Spudich, 1993; Spudich, 1993; Krah et al., 1994a,b; Seidel et al., 1995). The paradigm of adaptation in *E. coli* transduction partially applies to *H. salinarum*: a transient increase in the turnover of methyl groups has been observed in *H. salinarum* at the beginning and at the end of chemo- and photostimuli (Alam et al., 1989; Spudich et al., 1989). Indeed, methanol production during phototaxis in *H. salinarum* is much more similar to that observed during chemotaxis in *Bacillus subtilis* (Thoelke et al., 1989; Kirby et al., 1997). In the last few years several genes have been found in *H. salinarum* that encode for the homologous of the Che proteins (Rudolph and Oesterhelt, 1995, 1996; Rudolph et al., 1995). In par-

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ticular, the CheA histidine kinase and its substrate CheY have been characterized, and a putative phosphorylation site for CheA in the CheB sequence has been identified (Rudolph et al., 1995). Little is known about the CheR protein in this system. A possible four-state model for adaptation in *H. salinarum* has been proposed (Marwan et al., 1995), in which photocycling and methylation changes occur independently. In this scheme, light moves the system along photocycle events, whereas adaptation reactions move the system between methylated and unmethylated states.

During a saturating orange step, the system dwells mainly in the S_{373}-Htr-methylated state. This state is associated with a basal (unstimulated) reversal activity, whereas the transient states produced at the onset and the offset of the step are reversal-depressing and reversal-inducing, respectively. The balancing of these two signaling states makes *H. salinarum* insensitive to steady orange light. It is interesting, in light of the experimental results that will be reported below, that this scheme, even in its simplest version with fixed rates for methylation and demethylation, includes a summation process for repetitive pulses.

This scheme was also adopted to account for blue transduction (switching on a blue light pushes the system in the switching-inducing state), assuming that thermal decay of S_{373} is the slowest step (Marwan et al., 1995). However, this could only occur in the orange-adapted state and could not account for the specific interactions between orange and blue stimuli, observed by using a conditioning stimulus and one or two pulse test stimuli in sequence (Lucia et al., 1996, 1997). As will be discussed further on, these results cannot be explained simply by photocycling, and other problems arise from the existence of phototaxis mutants exibiting inverted responses (Olson et al., 1995).

A different scheme is reported in a very recent review (Hoff et al., 1997). Basically, the mechanism for adaptation is the same, but different signaling states are assumed to exist for orange and blue signals. Along this line, we thought it better to draw a complete scheme, in which all of the possible combinations of spectroscopic states of the photoreceptor and of methylation states of its transducer are considered (Fig. 1); this is a plausible scheme, because no evidence has been produced that the methylation state of the transducer(s) affects the photocycle time constants. Photoresponses should be interpreted, if possible, within the frame of these schemes, which are the reference context for current discussions on photobehavioral results.

A point to clarify in these schemes is the way in which different signals are integrated, or better, how the transduction system deals with the different signals coming from photo- and chemotransducers. In fact, both light and chemical signals control motile behavior and adaptation (Khan et al., 1995). As for light signals, considerable experimental evidence, obtained by a variety of experimental approaches, suggests that the basic mechanism for the activation of the receptor is the shuttling between two protein conformations (Yao et al., 1994; Rath et al., 1994; Bogomolni et al., 1994; Spudich and Lanyi, 1996; Jung and Spudich, 1996; Perazzona et al., 1996; Spudich et al., 1997; Zhang et al., 1997). However, specific interactions between signals coming from different photoreceptors or photoreceptor states have already been observed experimentally (Lucia et al., 1996, 1997). In this paper we present further results along this line, and discuss them in terms of the aforementioned models.

RATIONALE, EXPERIMENTAL PROTOCOL, AND SET-UP

The experiments reported here aim to follow the investigations on the "facilitation" observed when orange stimuli are applied in sequence (McCain et al., 1987; Lucia et al., 1996, 1997). Briefly, when a single orange pulse of 1 s or less is delivered after an orange step-down, its effect is to elicit reversals, whereas when delivered alone it does not affect

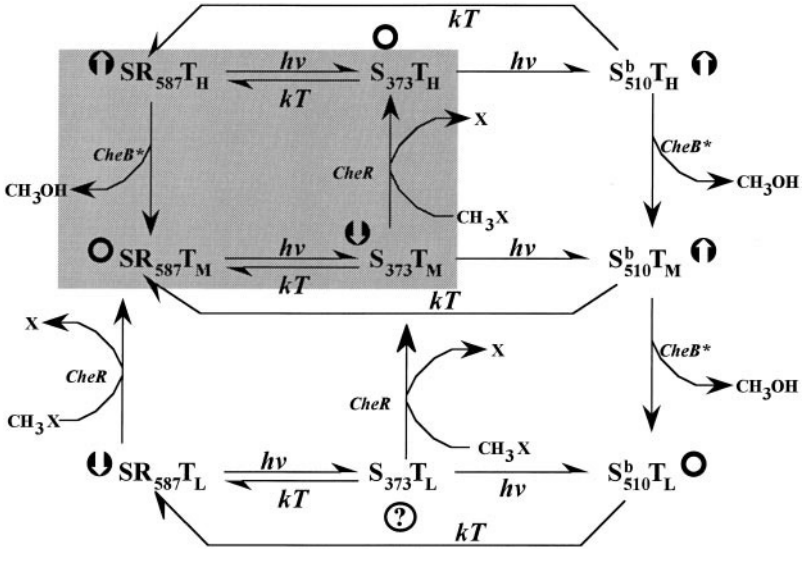

FIGURE 1 A nine-state model for phototaxis signaling by SR-I/HtrI. Photocycle intermediates are symbolized by their peak wavelength. T_H, T_M, and T_L stand for different methylation states (high, medium, and low) of the HtrI transducer. Histidine kinase activity of CheA associated with each photoreceptor state: ◐ = activated; ◑ = inhibited; ○ = basal; ? = unpredicted; kT = thermal decay; $h\nu$ = light stimulus of the appropriate wavelength. CH_3X = *S*-adenosyl-L-methionine; X = *S*-adenosyl-L-homocysteine.

swimming behavior (Lucia et al., 1996), or it induces a depression of the reversal frequency (McCain et al., 1987). It has been shown that this effect occurs only when the stimuli delivered after the conditioning orange step-down are pulses, because an orange step-up never elicits reversals under this condition (Lucia et al., 1996). Moreover, it has been shown that facilitation is not due to the photoresponse elicited by the conditioning stimulus; experiments were reported (Lucia et al., 1996) in which a conditioning stimulus of lower intensity elicits reversals without inducing facilitation, whereas in other experiments we also observed the reverse situation, when a reversal response to a pulse stimulus followed a weak response to the conditioning stimulus (unpublished results).

As a first experiment to get further information on the time course of this "facilitation," we used a very short series of orange pulses after an orange step-down. The result of this preliminary experiment (data not shown) showed that the facilitation sums up at each pulse.

At this point, we reasoned that repetitive stimuli, in the absence of a conditioning stimulus, are a useful tool for gaining information on the transduction processes in *H. salinarum*. Short trains of pulses were used to detect the transient behavior at the onset of a periodic stimulation, whereas experiments with long-lasting trains aimed to investigate the steady-state behavior.

The experimental set-up is here described in brief. A PC is equipped with a frame-grabber card (Matrox Pip 1024). The program running on this PC acquires a video image of the sample, stores in the computer memory the cell coordinates (within ~0.35 s, depending on the number of objects in the field), traces the cell trajectories, and counts the reversals. It also synchronizes (or is synchronized to) a second PC, an old computer used to deliver stimuli with each desired pattern. This avoids beating between the stimulus timing and the time required for the analysis of the acquired frames. Details on the apparatus for light stimulation are reported elsewhere (Lucia et al., 1996). The Flx15 mutant strain of *H. salinarum* (*bop*$^-$, *hop*$^-$, *sopI*$^+$, *sopII*$^+$, *htr-I*$^+$, *htr-II*$^+$) was used throughout this investigation. Cultures were grown as reported (Spudich and Spudich, 1982), and 3-day-old cells, unresponsive to blue-green light, were used in the experiments.

RESULTS

Fig. 2 shows the response to a short train of orange pulses. This pattern is obtained by triggering a finite train of orange pulses each time the program starts a run. A run consists of n frames, and during this time m pulses are delivered; then the system is allowed to adapt in the dark for 30 s before a new run begins. Typically, 10 runs are collected to draw a figure. In Fig. 2 *a* it is possible to observe that the response, which is absent after the first pulse, increases slowly and eventually reaches an asymptotic value. In similar experiments we always had the same trend, but sometimes two or three pulses were required before the first response appeared. A general observation is that the response to the last pulse is always higher and longer than the responses to the preceding pulses, as if they were partially inhibited by delivering the next pulse. Under particular conditions, it was not possible to observe responses during the train but only at its end, as in Fig. 2 *b*, where the period was too short to allow a response to develop before the rising phase of the next orange pulse, which depresses the reversal frequency.

Fig. 3 reports results obtained with an infinite series of orange pulses; data acquisition begins after several pulses, and is triggered by the onset of a pulse to synchronize the image analysis program. Three periods (10 s, 20 s, 50 s) were used; for the longest one no reversals were elicited, but still with a period of 20 s it is possible to observe a response. A remarkable observation is the shape of these responses: reported on a scale proportional to the period, they look quite similar.

Fig. 4 reports responses obtained by repetitively stimulating the sample with blue pulses. In Fig. 4 *a* the response to a train of 13 pulses is shown. It is clear that the response declines from the beginning, at variance with the results obtained with the same mode of stimulation using orange light. The response to infinite trains shown in Fig. 4, *b* and *c*, also shows a different trend when compared with the

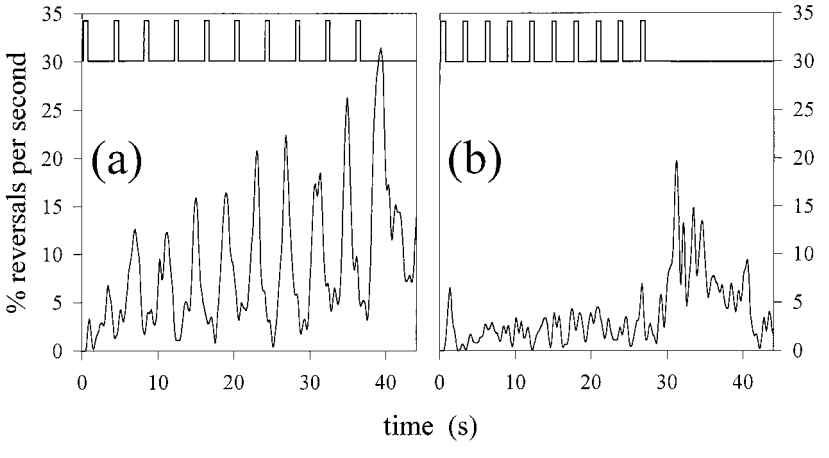

FIGURE 2 Responses to short trains (m = 10) of 0.6-s orange pulses; the pattern of stimulation is schematized in the upper part of each panel. (*a*) Time between pulses = 4 s. (*b*) Time between pulses = 3 s. Average number of cells: 190 in *a*, 240 in *b*. Maximum orange light intensity: K60 Balzer interference filter (600 ± 25 nm), 3×10^{17} photons cm^{-2} s^{-1}.

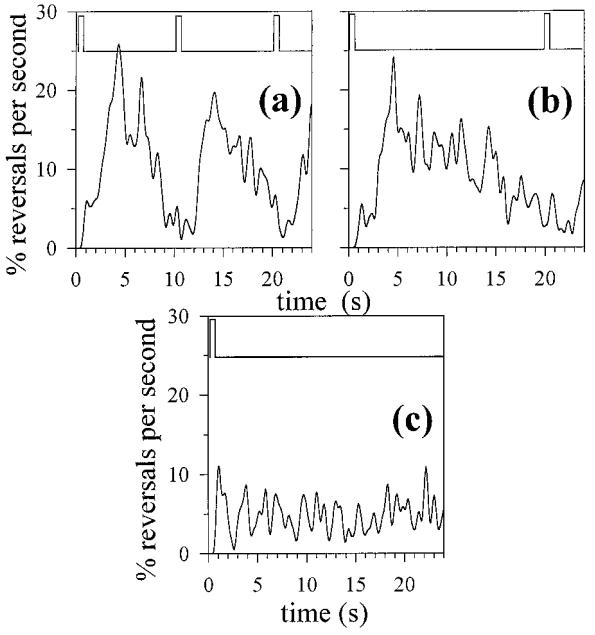

FIGURE 3 Responses to continuous trains of 0.6-s orange pulses: (*a*) with a 10-s period; (*b*) with a 20-s period; (*c*) with a 50-s period. The stimulation pattern is shown in the upper part of each panel. In *c* only a half-period is reported. Several cycles of pulsed stimulation were applied to the sample before data acquisition was begun at the onset of a pulse. Average number of cells: 260 in *a*, 300 in *b*, 210 in *c*. Maximum orange light intensity as in Fig. 2.

similar experiments on orange stimulation. It is clear, in fact, that with blue stimuli the highest response is obtained with the longest period.

CONCLUSIONS

Detailed insight into the photocycles of SR-I and SR-II is of primary relevance in this context. Some of the experimental results presented here and elsewhere reflect interactions between blue and orange transduction, both signals stemming from SR-I. The photocycle of this sensory pigment has been known for many years, and it is also known that the long-lived intermediate S_{373} is a signaling state, its concentration correlating with the behavioral responses to the offset of an orange light (Yan and Spudich, 1991; Marwan et al., 1995). Which is the signaling state for the blue signal is debated. Because the most likely candidate (S^{b}_{510}) is a shorter-lived intermediate (τ = 80 ms, compared to the 800 ms of S_{373}), the idea that blue transduction is due to pumping S_{373} back to the ground state has been considered (Marwan et al., 1995), a hypothesis that cannot be compatible with the fact that white light induces repellent responses (a more detailed discussion on this point can be found in Hoff et al., 1997).

However, also assuming the existence of a separate signaling state for blue transduction, it could be thought that a relevant interaction between orange and blue signals occurs at the photocycle level, with blue light destroying the signaling state of the orange signal. It is important to realize that this is not entirely true. By quantitatively solving the photocycle equations after a red-orange step-up in the presence and in the absence of a relatively high blue background, the difference in S_{373} concentration is found to be small (Fig. 5). Besides, white light induces repellent responses. Thus it is untenable that the inhibition of blue flashes with subsequent red stimuli is due simply to a decrease in S_{373}.

Let us now briefly summarize the results reported herein and in our previous papers on this issue (Lucia et al., 1996, 1997). Besides the after-effect of an orange step-down on following orange pulses, we observed

1. Over a sequence of blue pulses there is accumulation of something suppressing reversals (despite the fact that the direct effect of a blue pulse is to elicit them); on the contrary, a sequence of orange pulses accumulates facilitation. Moreover, the decline of the response after a pulse in a indefinite train depends on the frequency of stimulation (the decline is more rapid for more frequent pulses).

2. Blue pulses delivered after a conditioning stimulus (an orange step-down) are inhibited for a period up to 5 s; however, for longer delays their effect is increased (Lucia et al., 1996). Orange pulses, delivered after the conditioning stimulus, are able to elicit reversals in a range up to 10–20 s.

3. By delivering a blue pulse and an orange pulse in sequence a few seconds after the conditioning stimulus, it is possible to see that the inhibited blue pulse is still able to inhibit the response to the orange pulse (Lucia et al., 1997).

4. The response to an orange step-down is affected by blue light, because it is increased by a low level of blue light (Lucia et al., 1996).

Our results on repetitive stimulation with attractant light appear to be similar to those of Kirby et al. (1997) on *B. subtilis* chemotaxis; however, these authors report an increase in methanol production upon repetitive attractant chemostimulation at 90% receptor occupancy, whereas our results concern a motile response upon repetitive stimulation with (sub)threshold light pulses.

Is it possible to give an interpretation of at least some aspects of these results within the frame of excitation/adaptation models? Let us consider first the case of repetitive orange pulses. In this case the system will cycle in the gray part of Fig. 1. According to the simplest hypothesis, all of the rate constants are time-independent and are not affected in any way by the photoproducts. In this case, assuming rates of methylation and demethylation on the order of 0.05 s^{-1}, we obtain the results reported in Fig. 6 *A*, showing an increase over time of the receptor fraction in the $SR_{587}T_H$ state (the adapted state of the photoreceptor) upon repetitive orange stimulation. This figure, obtained by numerically solving the equations describing the model (the four-state model highlighted in gray in Fig. 1), shows that $SR_{587}T_H$ is high as long as the repetitive stimulation lasts, indicating that a high and almost constant frequency of reversals should be observed. This is at odds with the experimental findings. Therefore the accumulation process

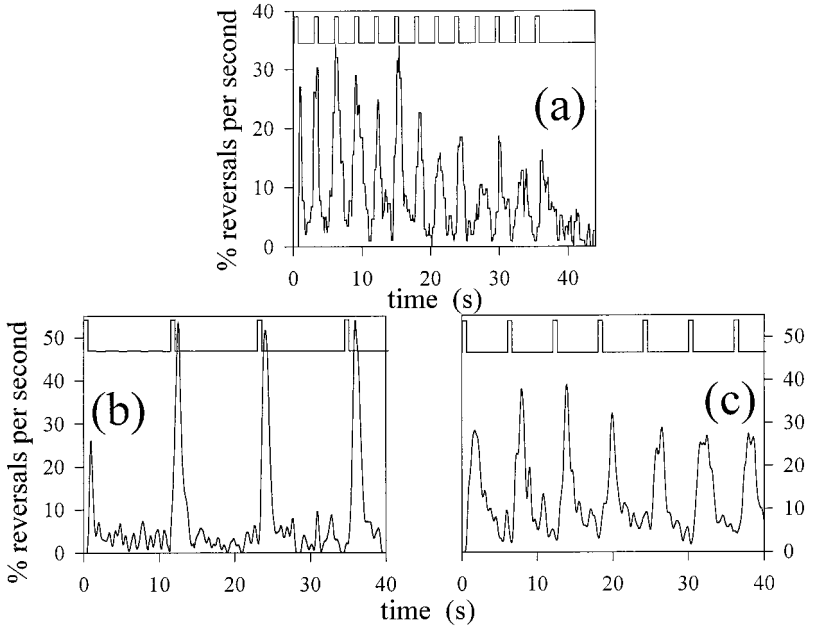

FIGURE 4 Responses to trains of 0.6-s blue pulses. Above: (*a*) Finite train of pulses (m = 13, distance between pulses = 3 s). Below: Continuous trains with periods of (*b*) 12 s and (*c*) 6 s. The stimulation patterns were applied and are schematized in the upper part of each panel as in Figs. 2 and 3. Average number of cells: 210 in *a*, 100 in *b*, 160 in *c*. Blue light intensity: K40 Balzer interference filter (400 ± 25 nm), 1.4×10^{16} photons cm^{-2} s^{-1}; background light (700 ± 25 nm): 10^{16} photons cm^{-2} s^{-1}.

inherent in the scheme of Fig. 1 is inadequate to account for the experimental results. We assumed as a further hypothesis that the demethylation rate (the CheB* concentration) is controlled by the average concentration of $SR_{373}T_M$, which in turn depends on the frequency of stimulation. In this way we expect to account for the shape of the responses to repetitive orange stimuli, which develops over a time scale tuned to the period of the stimulation.

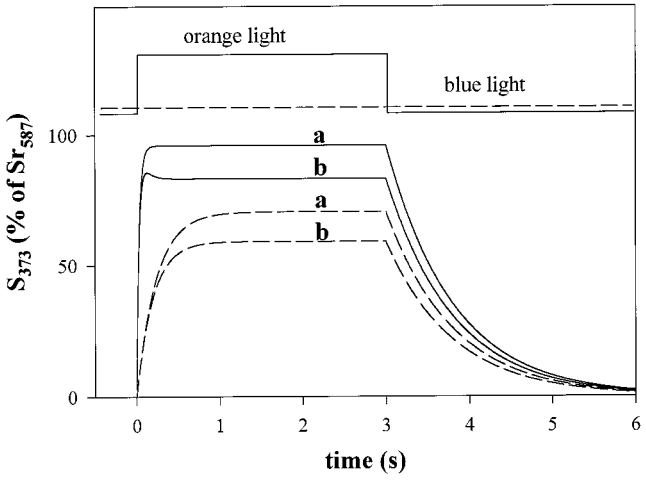

FIGURE 5 Calculated time courses of S_{373} after a 3-s orange light pulse in the absence (*a*) and in the presence (*b*) of a blue background. Continuous curves, orange light intensity = 3×10^{17} photons cm^{-2} s^{-1}; broken curves, orange light intensity = 3×10^{16} photons cm^{-2} s^{-1}. Blue light intensity = 6.6×10^{15} photons cm^{-2} s^{-1}. In the upper part of the figure, the orange light step (——) and the blue light level (– – –) are shown schematically. The time constant for the thermal decay of S_{373} was taken as 800 ms.

In mathematical terms this assumption is expressed by the following equation:

$$\frac{d[CheB^*]}{dt} = -a[CheB^*] + b[S_{373}T_M]$$

where a is the rate constant for spontaneous decay and b is an apparent first-order kinetic constant for CheB activation. Including this equation in the set describing the four-state model, we obtained the results reported in Fig. 6 *B*, which look satisfactorily similar to the experiments reported in Fig. 3, *a* and *b*.

The specificity of the interactions between blue and orange pathways (Lucia et al., 1996, 1997) and the variation of CheB activity, accounting for the results of repetitive stimulation—in particular, for tuning the response decay with the stimulus period—are probably connected to the existence in *H. salinarum* of distinct signals, not directly dependent on CheA activation, stemming from $SR_{373}T_M$. It is worth noting that the paradigma of signal transduction in *E. coli* involves only a few chemical species, in particular only two (CheR and CheB) that control the methylation/demethylation of the receptors. In other bacteria the transduction pathways seem more complex and involve more methylation-connected proteins. For instance, the MCP methylation rate in *B. subtilis* is modulated by two additional chemotaxis proteins, CheD and CheC (Rosario et al., 1995; Rosario and Ordal, 1996), whereas in *Rhodobacter sphaeroides* several varieties of CheA, CheR, CheW, and CheY have been found (Hamblin et al., 1997).

The hypotheses described above account for point 1. As for points 2 and 3, assuming that, after an orange step-down, activated CheB demethylates $S^b_{510}T_H$ at high speed would explain why the effect of the blue flash does not result in reversal activity, but is still present as an antagonist to the

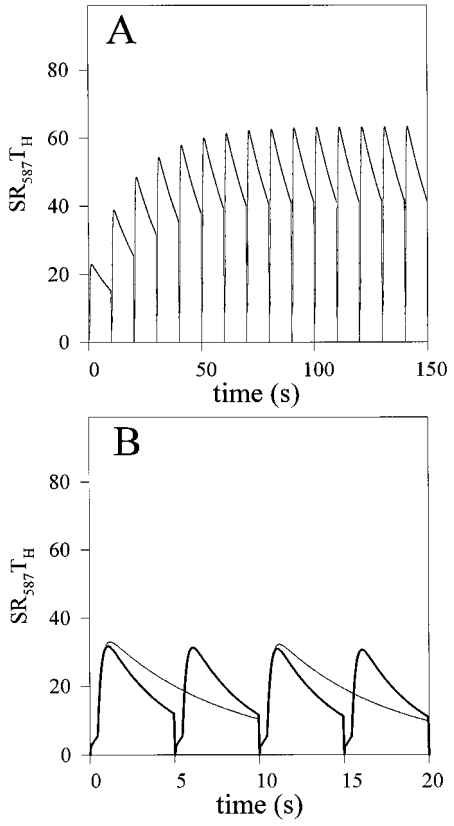

FIGURE 6 Calculated time courses of $S_{587}T_H$ for the four-state model highlighted in gray in Fig. 1. The total amount of SR-I is normalized to 1000; the time constant for the thermal decay of S_{373} was taken as 800 ms. (*A*) Pulsed stimulation every 10 s, starting from $t = 0$; $K_b = K_f = 0.05\ s^{-1}$. (*B*) Pulsed stimulation every 5 s (*thick line*) and every 10 s (*thin line*); $K_f = 0.05\ s^{-1}$, whereas K_b depends on the concentration of $S_{373}T_M$ according to the equation reported in the text with the parameter values $a = 0.01\ s^{-1}$, $b = 5 \times 10^{-5}\ s^{-1}$.

orange signal (some $S_{587}T_L$ will be present). To test this interpretation, critical experiments can be designed starting from the model in Fig. 1, which we consider as a tentative and improvable guide to future work.

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