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ORIGINAL PAPER

Dynamics of electron transfer from high-potential cytochrome *c* to bacteriochlorophyll dimer in photosynthetic reaction centers as probed using laser-induced temperature jump

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Abstract Laser-induced temperature jump experiments were used for testing the rates of thermoinduced conformational transitions of reaction center (RC) complexes in chromatophores of *Chromatium minutissimum*. The thermoinduced transition of the macromolecular RC complex to a state providing effective electron transport from the multiheme cytochrome *c* to the photoactive bacteriochlorophyll dimer within the temperature range 220–280 K accounts for tens of seconds with activation energy 0.166 eV/molecule. The rate of the thermoinduced transition in the cytochrome–RC complex was found to be three orders of magnitude slower than the rate of similar thermoinduced transition of the electron transfer reaction from the primary to secondary quinone acceptors studied in the preceding work (Chamorovsky et al. in Eur Biophys J 32:537–543, 2003). Parameters of thermoinduced activation of the electron transfer from the multiheme cytochrome *c* to the photoactive bacteriochlorophyll dimer are discussed in terms of cytochrome *c* docking onto the RC.

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Abbreviations

RC	Reaction center
P	Photoactive bacteriochlorophyll
Q_A	Primary quinone acceptor
Q_B	Secondary quinone acceptor

Introduction

Bacterial photosynthetic reaction center (RC) includes three integral membrane polypeptide subunits bound to the chromophore groups mediating the processes of the photoinduced primary charge separation (Allen et al. 1987; Deisenhofer et al. 1995).

Bacterial RC is a unique example of a macromolecular system whose activity is closely related to conformational mobility. The effect of conformational mobility on specific electron-transport activity was studied at various stages of electron transport. During the initial stages of charge separation in the complex of porphyrin molecules of RC, the intramolecular dynamics primarily provide fast relaxation processes in the immediate vicinity of electron transport cofactors, thereby providing effective temporal stabilization of separated charges (Paschenko et al. 1998; Volk et al. 1998). According to an FTIR study, further reduction of the primary quinine acceptor Q_A gives rise to large changes in the bonding interactions between the quinone carbonyls and the RC protein. Upon charge separation and stabilization, a new configuration of the quinone is achieved. Accompanying the reduction of

Q_A, specific microconformational changes of amino acid side chains and/or of the polypeptide backbone occur, probably in the vicinity of Q_A, although electrostatic effects at more distant sites should be considered (Breton et al. 1994). Electron transfer in the system of quinone acceptors Q_A–Q_B of purple bacteria RC is substantially associated with protein microconformational dynamics, because it provides some important reactions including protonation of certain amino acids during relaxation processes coupled to temporal stabilization of the electron. (Miksovska et al. 1995; Rabenstein et al. 2000). These processes in the protein may depend on binding of specific molecules (e.g., lipids) to specific sites in the RC. This is illustrated by experimental results of incorporation of isolated RC into micelles of membrane lipids. It is conceivable that the effect of lipid environment is mediated by modification of the intraprotein network of hydrogen bonds of RC (Nagy et al. 2004). It was shown in our experiments that the state of the network of hydrogen bonds of RC might indeed have a significant effect on the relaxation processes associated with charge separation in RC, thereby affecting the electron transport rate (Paschenko et al. 1998). The time dynamics of the relaxation processes and microconformational changes may cause more pronounced structural changes in the macromolecule (macroconformational transitions). For example, X-ray scattering analysis of RC crystals revealed that freezing of conformational mobility of RC in the light and in the dark gave rise to two different positions of the secondary quinine Q_B in the RC protein structure. The positions differ from each other by a 5 Å shift and a 180° turn of the quinone (Stowell et al. 1997).

In addition to the three integral subunits, RC complexes of certain species of photosynthesizing purple bacteria (*Rhodopseudomonas viridis, Chromatium vinosum, C. minutissiimum, Ectothiorhodospira shaposhnikovii*, etc.) contain a multiheme cytochrome subunit exposed to the water phase (Deisenhofer et al. 1995). The quantum efficiency of the primary processes of charge separation in bacterial RC does not decrease upon cooling to cryogenic temperature (Arnold and Clayton 1960; Clayton 1980). On the other hand, temperature decrease has a significant effect on the reaction of electron transfer from the primary to the secondary quinone acceptors (Dutton and Prince 1978; Rubin et al. 1987) and from the secondary to the primary donor (cytochrome *c* and bacteriochlorophyll dimer, respectively). Temperature dependences of electron transfer rates in biological systems provide essential information about physical mechanisms of biological processes. Temperature has a significant effect on the reactions of electron transfer from the multiheme cytochrome *c* to the photooxidized dimer of RC bacteriochlorophyll (De Vault and Chance 1966; Dutton and Prince 1978; Rubin et al. 1987). Studies on temperature dependence of electron transfer rates in biological systems provide important insight into physical mechanisms of biological processes. It should be emphasized that it was the temperature dependence of the reaction of electron transfer from cytochrome *c* to RC bacteriochlorophyll dimer in the purple photosynthetic bacterium *Chromatium* that suggested to Chance the pioneering idea of electron tunneling in biological systems (De Vault and Chance 1966). Experimental data on the temperature dependence of the cytochrome *c* photooxidation in photosynthetic bacteria gave rise to a number of theoretical models (Bixon and Jortner 1986, 1988; Kuhn 1986; Knapp and Fischer 1987). The temperature dependence of tetraheme cytochrome photooxidation in *Rhodopseudomonas viridis* was studied by Mathis et al. (Ortega and Mathis 1993; Ortega et al. 1998). The rate of the photooxidation reaction was shown to be nearly temperature-independent within the range from 294 to 8 K. The freezing at low temperatures of cytochrome structural reorganization was hypothesized. Virtual temperature independence of the rate of photooxidation of high-potential cytochrome *c* was observed in *Ectothiorhodospira shaposhnikovii* (Chamorovsky et al. 1980, 1986) and *Chromatium minutissimum* (Rubin et al. 1989). The temperature dependence of the photooxidation of low-potential cytochrome *c* in *Chromatium minutissimum* was found to be biphasic (temperature-dependent and temperature-independent phases). Such a temperature pattern was explained by a model of molecular dynamics of the RC protein. The temperature dependence of electron transfer from multiheme cytochrome *c* to RC bacteriochlorophyll dimer in the RC of the purple photosynthetic bacterium *Rhodopseudomonas sulfoviridis* was studied within the temperature range from 296 to 40 K (Kotel'nikov et al. 2002). The kinetics of the reaction was interpreted using a model of temperature-independent vibrational and temperature-dependent diffusion components of protein relaxation.

Variation of the rate of temperature changes provides a promising experimental approach to studies of the role of conformational dynamics of phototransformation proteins and charge transfer mediated by these proteins. This approach can be used not only to trap nonequilibrium protein states involved in charge transfer, but also to assess kinetic and thermodynamic parameters of protein transition between different conformational substates affecting the rates of functional processes (Zubov et al. 1983; Parot et al. 1987; Kirmaier

and Holten 1990; McMahon et al. 1998; Xu and Gunner 2001, 2002). In one of the first works addressing this problem, light-induced structural changes of photosynthetic RC protein were experimentally observed by rapid immersion of RC preparations of *Rhodobacter sphaeroides* into liquid nitrogen in the light and in the dark (Kleinfeld et al. 1984). The same approach was later applied to RC crystals. In combination with X-ray diffraction analysis, this revealed distinct changes in position and orientation of the secondary quinone acceptor Q_B in the RC interior upon Q_B photoreduction (Stowell et al. 1997).

In addition to thermoinduced equilibrium states, the temperature jump method allows dynamic parameters of transitions between the thermoinduced states to be studied. The effect of nonstationary thermal perturbation (temperature jump induced by infrared laser pulse) on electron transfer in the complex of quinone acceptors of bacterial photosynthetic RC was studied in the preceding work (Chamorovsky et al. 2003). Kinetic and thermodynamic characteristics of thermoinduced transition from inactive (frozen) to active RC quinone complex states were estimated. It seemed interesting to extend this approach to the reaction of electron transfer from the multiheme cytochrome to bacteriochlorophyll in purple bacteria.

The goal of this work was to study the temperature-induced effect on electron transfer from the multiheme cytochrome *c* to photoactive bacteriochlorophyll dimer in *Chromatium minutissimum* and compare the resulting kinetic and thermodynamic characteristics of thermoinduced transitions in the site of the electron-transfer chain with those in the quinone complex of the chain estimated in the preceding work (Chamorovsky et al. 2003).

Materials and methods

Chromatophores and isolated pigment–protein RC complexes from the photosynthesizing bacterium *Chromatium minutissimum* were used. Bacterial cells were grown, and chromatophores and RC preparations were isolated as described in the literature (Zakharova and Churbanova 2000). Chromatophores and isolated RC preparations were suspended in Tris–HCl or phosphate buffer containing 0.2 M sucrose and 5 mM $MgSO_4$ (pH 7.0). Some experiments were performed using air-dry films or water–glycerol suspensions of chromatophores and isolated RC preparations. Air-dry films were used in experiments with single laser flashes, and water–glycerol suspensions of chromatophores and isolated RC were used in experiments with trains of laser flashes, because trains of laser flashes might cause local damage to thin dry films.

Photoreactions in chromatophores and RC preparations of purple bacteria were induced by the second harmonic of a pulsed YAG:Nd laser (wavelength, 530 nm; pulse duration, 15 ns) or an incandescent lamp (wavelength, >700 nm; exposure time, 0.5–1 s). Redox reactions of the RC bacteriochlorophyll dimer (P870) were monitored as absorption changes at the Soret band (430 nm). Redox reactions of cytochrome *c* were monitored at the gamma-band at 425 nm. Spectral bands of monitoring light were isolated using corresponding interference filters.

Radiation of a YAG:Er^{2+} pulsed laser (wavelength, 2.94 μm) or a continuous-mode CO_2 laser (wavelength, 10.6 μm) was used to induce pulsed heating (temperature jump). Measuring the inverse current of a KD-103 diode attached to the sample tested monitored the initial steady-state temperature.

The maximum magnitude and kinetics of heating induced by the YAG:Er^{2+} laser were calculated from the laser radiation intensity, extinction coefficients of sample and support at the laser radiation wavelength, and heat capacity of sample and support. The kinetics of heating induced by the continuous-mode CO_2-laser was mainly determined by the switching rate of the mechanical shutter. The calculated half-time of the temperature increase induced by YAG:Er^{2+} laser or CO_2 laser was 150–400 μs or 1 s, respectively. The amplitude of the laser-induced temperature increase was varied over a broad range by changing the laser radiation intensity (using glass filters).

It should be noted that the results of these calculations are uncertain in some cases, because they depend on such uncertain factors as distribution of laser radiation over the cross-section of the laser beam and laser radiation distribution between sample and support. Therefore, it was reasonable to measure the laser-induced temperature changes experimentally. Laser-induced temperature changes were calibrated using absorption changes of the initial form of bacteriorhodopsin (bR-570) or fluorescence of rhodamine *B*. It is well known that the Arrhenius plot of the rate of dark recovery of bR-570 followed flash-induced excitation of the photocycle is a linear function. Because the recovery time of bR-570 (milliseconds at room temperature) is much shorter than the relaxation time of the temperature jump induced by CO_2- rather than YAG:Er^{2+}-laser, the absorption change kinetics of bR-570 can be used as a low-inertia sensor of the temperature changes induced by the CO_2-laser. An example of the kinetic profile of the thermal pulse

generated at the CO_2-laser pulse energy of 8.3 J is shown in Fig. 1a. (For more detail, see Chamorovsky et al. 2003.)

The optical settings used in calibration were identical to those used in further experiments with chromatophores or photosynthetic RC preparations.

The kinetic profiles of thermal pulses generated by the YAG:Er^{2+}-laser in thin films (10–20 μm) of water–glycerol suspensions were calibrated similarly using either faster intermediates of the bacteriorhodopsin photocycle or fluorescence of rhodamine *B*. The results

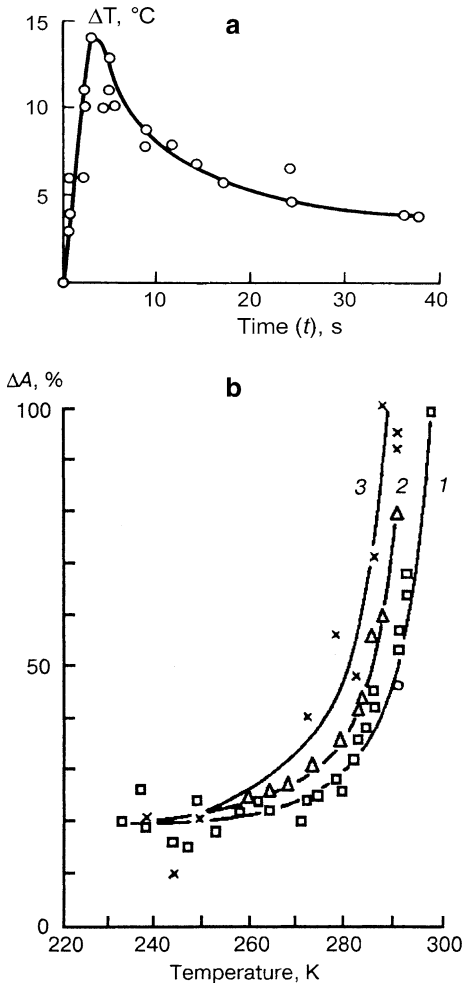

Fig. 1 **a** Kinetic profile of thermal pulse generated by CO_2-laser radiation (wavelength, 10.6 μm; flash duration, 1 s; radiation energy, 8.3 J). Adapted from Chamorovsky et al. (2003). **b** Temperature dependence of the amplitude of the photoinduced absorption changes at 425 nm associated with cytochrome *c* oxidation in the *C. minutissimum* chromatophores: *1* control (without thermal pulse); *2* against the background of thermal pulse of CO_2-laser with amplitude 17°; *3* against the background of thermal pulse of CO_2-laser with amplitude 33°. Abscissa in curves *2* and *3* corresponds to the initial temperature of the experimental sample before application of the thermal pulse. Experimental samples contained 70 vol% glycerol. Photoactivation duration, 0.5 s

of these measurements, as well as theoretical simulation, showed that both the leading and trailing edges of the thermal pulse in this case are much faster than in case of the CO_2-laser. According to the pulse energy, the rise-time of the thermal pulse generated by the YAG:Er^{2+}-laser ranged from 150 to 400 μs, whereas the temperature relaxation kinetics contained two stages. The half-time of the temperature decay (temperature relaxation to the level of 50%) was about 1 ms, whereas the time of complete relaxation to the initial level was several tens of milliseconds.

In case of dry films of chromatophores (thickness, 10–20 μm), the rise-time of the thermal pulse generated by the YAG:Er^{2+}-laser was approximately the same (150–400 μs), whereas the half-time of temperature relaxation and time of virtually complete relaxation to the initial level were about 10 ms and several tens of seconds, respectively (Zubov et al. 1983; Chamorovsky et al. 2003). Thus, the use of the pulsed YAG:Er^{2+}-laser and the quasipulsed CO_2-laser (continuous generation modulated with a mechanical shutter) allowed study of the kinetics of both fast (milliseconds) and slow (tens of seconds) thermoinduced processes in biological membranes.

Kinetics of photoinduced electron transport reactions induced in chromatophores and isolated RC preparations by steady-state light were measured using a differential single-beam spectrophotometer as described earlier (Rubin et al. 1987).

Results and discussion

In the preceding work (Chamorovsky et al. 2003) laser-induced temperature jump was used for testing the rates of thermoinduced conformational transitions of RC complexes in chromatophores and isolated RC preparations of photosynthesizing purple bacteria. Electron transfer from the primary to secondary quinone acceptors was used as a probe of the electron transport efficiency. The use of IR laser flashes of different duration allowed the characteristic time of the thermoinduced transitions in the quinone complex to be estimated as less than several tens of milliseconds. In this work we applied this approach to the cytochrome *c* segment of the RC electron-transport chain. The electron-transport activity of cytochrome *c* was monitored using the amplitude of the photoinduced absorption decrease at 425 nm. The photoinduced absorption changes at 425 nm contain components associated with photoreactions of cytochrome *c* (maximum at 425 nm) and photoactive RC bacteriochlorophyll (maximum at 430 nm). The absorption changes at

425 and 430 nm are very close, and interference filter bandwidth does not allow them to be resolved from one another unambiguously using spectral analysis alone. However, photoinduced absorption changes associated with photoreactions of cytochrome *c* and RC bacteriochlorophyll at 425 nm are negative and positive, respectively. The amplitude of the photoinduced absorption changes at 425 nm proportional to the fraction of the cytochrome *c* molecules capable of being photooxidized is temperature-dependent. The amplitude of the high-potential cytochrome *c* oxidation abruptly declines near 200 K (Ortega et al. 1998; Chamorovsky et al. 1980; Rubin et al. 1989). Various mechanisms of low-temperature inhibition of electron transport reactions in bacterial RC are discussed in the literature. For example, models of the temperature-induced shift of redox levels (Case and Parson 1971; Kaminskaya et al. 1990) or deceleration of conformational mobility of protein subunits of RC (Rubin et al. 1990) have been suggested. Perhaps, experimental data obtained using laser-induced T-jump may help to distinguish between these models.

Exposure to a thermal pulse was shown to induce partial recovery of the amplitude of the photoinduced absorption changes at 425 nm proportional to the fraction of the photoactive cytochrome *c*. If the thermal pulse energy was maintained at an approximately constant level, the extent of the recovery was found to increase upon increasing the pulse duration. It was found empirically that among single laser flashes tested in this work, the longest duration of the thermal pulse (7 s) was observed in case of radiation generated by the CO_2-laser (wavelength, 10.6 μm; flash duration, 1 s). The kinetic profile of the pulse is shown in Fig. 1a. The measurement of the laser-induced T-jump pulse profile was described in more detail in the preceding work (Chamorovsky et al. 2003). However, even such thermal pulses were incapable of inducing complete recovery of cytochrome *c* photooxidation inhibited by low temperature. The temperature dependence of the amplitude of the photoinduced absorption decrease at 425 nm caused by cytochrome *c* oxidation in water–glycerol suspension of the *C. minutissimum* chromatophores is shown in Fig. 1b, curve *1*. The measuring error of this data was 10–15%, which is typical of biophysical experiments. After the chromatophore preparations had been cooled to the required temperature, they were exposed to the laser thermal pulse. Two series of experiments were performed with laser flash energy 11.5 and 23 J (temperature jump amplitude 17 and 33 K, respectively). In the two experimental series, the photoinduced electron transfer reactions of cytochrome *c* oxidation were probed 7 s after the infrared laser flash. The results of the first and second experimental series are shown in Fig. 1b (curves *2* and *3*, respectively). It follows from Fig. 1b that although thermal pulses increased the amplitude of the photoinduced signals, the increment of the thermoinduced increase was less than expected (17 and 33 K in the first and second experimental series, respectively). The measuring error in these experiments was 10–15%.

It should be noted that the increment of the thermoinduced increase in the photoinduced signal was found to be temperature-dependent. Indeed, the increment decreased upon decreasing the initial temperature of the sample, and within the temperature range from 253 to 243 K the thermal pulse induced only an insignificant increase in the photoinduced signal amplitude. It was noted above that among single laser flashes tested in this work, the longest duration of the thermal pulse (7 s) was inherent in the CO_2-laser. Nevertheless, it is safe to suggest that the lifetime of the thermal pulse is insufficient to attain the conformational equilibrium between the multiheme cytochrome *c* subunit and photoactive bacteriochlorophyll dimer providing high-efficiency electron transfer in this system.

Other experimental settings were used to increase the thermal pulse duration and to test the kinetics of the thermoinduced implementation of the conformational equilibrium between the multiheme cytochrome *c* subunit and photoactive bacteriochlorophyll dimer. The YAG:Er^{2+}-laser was set in the spontaneous generation mode with flash repetition frequency 3 Hz, flash duration 150 μs, and flash energy 0.5 J. When the required sample temperature was reached, it was stabilized by attenuating the laser radiation energy with KS glass filters. The temperature stability was judged from invariability of sample absorption. After a steady-state temperature of the sample was attained and maintained stable for a time interval *t*, the sample was exposed to actinic light, and the amplitude of photoinduced oxidation of cytochrome *c* was recorded. The time diagram of such experiment is illustrated in Fig. 2. Kinetic curves of cytochrome *c* absorption changes induced by continuous light at different temperatures are shown at the top of Fig. 2. Actinic light on and off induced photooxidation of cytochrome *c* and its dark reduction, respectively. Trace 1 in Fig. 2 top shows a downward change in the absorption before the actinic light is switched off. This phenomenon is presumably due to transition of the electron-transport chain to a photostationary state. Such transitions to stationary states were studied in various photosynthetic systems using mathematical modeling (Rubin et al. 1987). Detailed discussion of this problem is beyond the scope

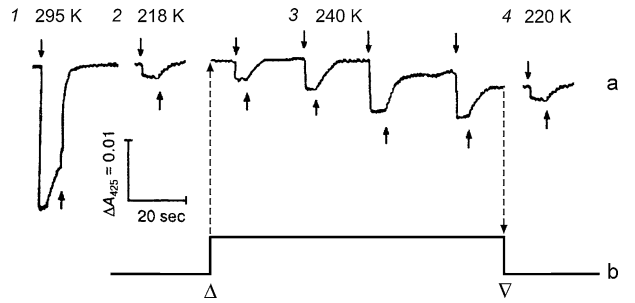

Fig. 2 Kinetics of photoinduced absorption changes at 425 nm associated with cytochrome *c* oxidation in air-dry films of *C. minutissimum* chromatophores. Thin *downward and upward arrows* indicate the moments of actinic light on and off, respectively. *Triangles with the vertex up and down* indicate the moments of the thermal radiation of YAG:Er-laser on and off, respectively (explanation in text)

of this work. The initial amplitude of the light-induced absorption changes (295 K) declined with decreasing temperature (218 K). The time profile of the temperature pulse formed using a train of YAG:Er^{2+}-laser flashes and KS glass filters is schematically shown at the bottom of Fig. 2. Although there were short-term transition processes at the leading edge of the temperature pulse, the trailing edge of the pulse was somewhat delayed; these deviations from the rectangular shape had little if any effect on the interpretation of the results discussed later. Therefore, for the sake of illustrative presentation, the thermal pulse used in our experiments to a first approximation can be regarded as rectangular. The time interval required to attain the half-maximum amplitude of the signal of photoinduced oxidation of cytochrome *c* was measured plotting the dependence of the cytochrome oxidation amplitude on the time of exposure to the thermal pulse. This curve is shown in the inset to Fig. 3. The Arrhenius plot of the temperature dependence of the characteristic time interval required to attain the half-maximum amplitude of the signal of photoinduced oxidation of cytochrome *c* within the temperature range from 220 to 280 K is shown in Fig. 3. The slope of the curve corresponds to the activation energy of the corresponding conformational transition, 16 kJ/mol. Both kinetic and thermodynamic characteristics of this transition are typical of either displacement of large fragments of macromolecules or conformational changes of macromolecules as a whole. To a first approximation, this conclusion is in line with reported theoretical estimates (Miyashita et al. 2005) for the electron transfer between cytochrome c_2 and reaction center from photosynthetic bacteria. It should be noted that studies of the temperature dependence of the photoinduced electron transport from the multiheme cytochrome *c* to the photoactive bacteriochlorophyll dimer in seven mutant strains of *Rps. viridis* with the amino acid residue Tyr-62 substitution (Tyr-62 is located in RC between the multiheme cytochrome *c* and photoactive bacteriochlorophyll dimer) suggested that the low-temperature inhibition of this reaction depends on the conformational transition of the protein moiety of RC and is mediated by a network of water molecules (Ortega et al. 1998). Different behaviors of wild type and mutant reaction centers were attributed to differences in a network of water molecules, the freezing of which may block structural reorganizations associated with cytochrome oxidation, in the wild type but not in the mutant (Ortega et al. 1997).

It should also be noted that rates of many biochemical reactions are changed without accompanying conformational translations. However, the magnitude and kinetic characteristics of the processes studied in this work imply involvement of temperature-induced conformational transitions of proteins. Perhaps it is worthwhile comparing parameters of these transitions with parameters of cytochrome *c* docking onto the RC. The electrostatic interactions involved in the dynamics of docking of cytochrome onto the RC examined by double mutant studies revealed that the interaction energies are approximately inversely proportional to the distances between interacting charges and indicate that specific electrostatic interactions facilitate docking of the cytochrome onto the RC in a configuration optimized for both binding and electron transfer (Tetreault et al. 2002). The importance of conformational dynamics and electrostatic interactions between cytochrome c_2 and reaction centers in enhancement of the electron transfer reaction in photosynthetic bacteria was demonstrated in a theoretical study (Miyashita et al. 2004).

It is also possible that the results observed in our experiments are attributed to larger scale rearrangement in the supramolecular assembly of the complexes. The slow kinetics of these processes can be regarded as evidence in favor of this suggestion.

Thus, to summarize the results of the study and the previous work (Chamorovsky et al. 2003), it should be noted that the rate of the thermoinduced conformational transition providing effective electron transfer between redox centers embedded into the integral RC protein structure (Q_A and Q_B; millisecond time range) is significantly higher than the rate of similar thermoinduced conformational transition providing electron transfer between redox centers located at peripheral and integral subunits (multiheme cytochrome *c* and bacteriochlorophyll dimer, respectively; tens of seconds). Assuming that the electron tunneling distances are similar, the three-order-of-magnitude difference between the rates of temperature-induced restoration of electron transport

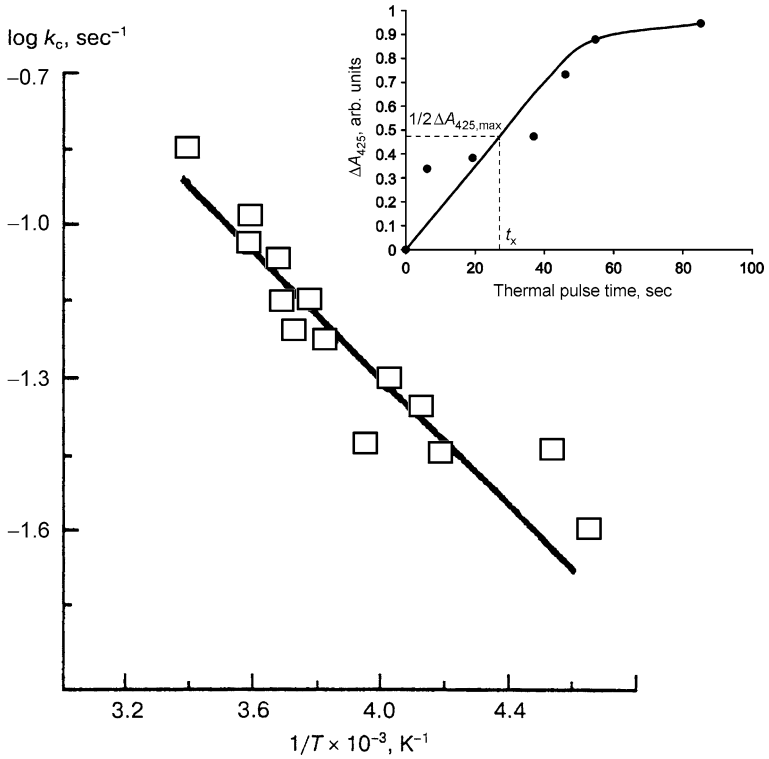

Fig. 3 Arrhenius plot of the temperature dependence of the rate constant (k_c) of the thermoinduced conformational transition of RC complex to the state with photoactivated electron transfer from cytochrome *c* to bacteriochlorophyll dimer. *Inset* dependence of the cytochrome oxidation amplitude at 425 nm on the time of sample exposure to the thermal pulse

activity at the acceptor and donor (cytochrome) sides of the chain would imply that the molecular groups involved in the processes of restoration have substantially different sizes. The kinetic and thermodynamic characteristics of the transition involving cytochrome *c* and bacteriochlorophyll dimer imply that the molecular model of corresponding process should be based on large-scale conformational dynamics (e.g., association/dissociation of the cytochrome subunit of RC).

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