Biochemistry (Moscow), Vol. 66, No. 7, 2001, pp. 769-773. Translated from Biokhimiya, Vol. 66, No. 7, 2001, pp. 947-951.

Recombination Reduction of Photooxidized Cytochrome *c* in Reaction Centers of *Rhodopseudomonas viridis* at Low Temperature

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Received December 5, 2000
Revision received March 2, 2001

Abstract—The temperature dependence of dark reduction of photooxidized cytochrome *c* was studied in isolated preparations of *Rhodopseudomonas viridis* reaction centers. Within the range from room temperature to ~260 K this process was found to be mediated by thermal diffusion of exogenous donor molecules, whereas at lower temperatures photooxidized cytochrome is reduced as a result of indirect recombination with photoreduced primary quinone acceptor. Kinetic simulation allowed certain thermodynamic characteristics of this reaction to be calculated. To the first approximation, these characteristics correlate with the estimates obtained from the results of direct redox titration.

Key words: photosynthetic reaction center, cytochromes, electron transport, recombination, purple bacteria

Photosynthetic reaction centers (RC) of the purple bacterium *Rhodopseudomonas* (*Blastochlorii*, according to the new classification) *viridis* is a nearly ideal object for studying physical mechanisms of electron transport reactions in biological systems. Indeed, the three-dimensional structure of RC has been determined with high atomic resolution by X-ray diffraction [1], whereas their functional activity can be tested by various spectral techniques within a broad range of temperature and time scales [2-4].

In addition to three integral RC subunits found in virtually all species of purple bacteria, the RC complexes from *Rps. viridis* also contain a four-heme cytochrome *c*, which donates electrons to the photooxidized dimer of RC bacteriochlorophyll [1]. The mechanism of electron tunneling in biological systems was proposed by Chance and De Vault to describe the temperature dependence of this reaction in the bacterium *Chromatium vinosum* [5]. In recent years, a large body of new data have been obtained about the temperature dependence of the rate of photooxidation of the four-heme cytochrome *c* in wild-type strains and site-directed mutants of *Rps. viridis* [6, 7]. However, there are virtually no data on the broad-range temperature dependence of the reaction of dark reduction of photooxidized cytochrome *c* in the *Rps. viridis* RC complexes. Although this is particularly true in case of steady-state photoexcitation, these are the conditions allowing the information about distribution of electron flows in the system to be obtained. These experiments can also provide additional important information about mechanisms of electron transport reactions.

The goal of this work was to study the effect of temperature variation from room to cryogenic on the reaction of dark reduction of photooxidized cytochrome *c* in isolated RC complexes of *Rps. viridis* under conditions of steady-state photoexcitation.

MATERIALS AND METHODS

Pigment–protein complexes of RC were isolated from chromatophores of *Rps. viridis* using hydroxyapatite chromatography by a modification of the method described in [8]. The main difference from [8] was that 1% rather than 5% lauryl dimethylamine oxide (LDAO) was used to solubilize chromatophore membranes.

Isolated RC complexes were suspended to the final concentration of ~7 μM in 10 mM sodium phosphate buffer (pH 7.0) containing 0.05% LDAO. Immediately before experiments the redox potential of the reaction medium (1 ml of RC suspension) was poised to fall

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within the range from +200 to +400 mV. 2,6-Dichlorophenol indophenol (DCPIP) (~100 μM) was used as a redox mediator. Potassium ferricyanide and sodium ascorbate were used as titrating agents. A Pt wire measuring electrode (diameter, 1 mm) and an Ag/AgCl reference electrode (Microelectrodes Inc., USA) were used to measure redox potential. An Accumet Basic digital pH-meter (Fisher Scientific, USA) was used. Low-temperature measurements were performed using RC suspension containing 60 vol. % glycerol.

Kinetics of photoinduced electron transport reactions induced in isolated RC preparations by steady-state light (λ > 640 nm; 180 W/m^2) were measured using a differential single-beam spectrophotometer equipped with a cryostat as described in [9]. The dark recombination between the photooxidized bacteriochlorophyll dimer and photoreduced primary quinone acceptor (Q_A) was measured at 980 nm using the same spectrophotometer and photoactivation with a short light pulse generated by an ISSh-100 3M stroboscopic flash lamp (pulse duration, 10 μsec; spectral range, 400-600 nm; pulse energy, 9 mJ). Electron transfer from Q_A to the secondary quinone acceptor Q_B was inhibited, if necessary, by the addition of 10 mM *o*-phenanthroline.

Temperature was measured using a Cole Parmer Instrument Co. digital thermometer (USA) with a thermocouple inserted in the low-temperature cuvette with sample.

Absorption spectra were measured using a Hitachi-557 spectrophotometer (Japan).

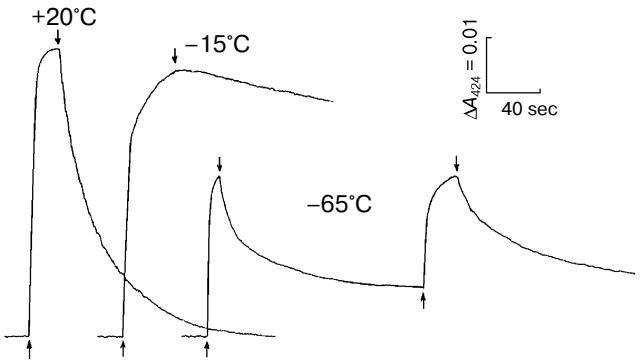

Fig. 1. Light-induced absorption changes of cytochrome *c* at 424 nm in RC complexes of *Rps. viridis* measured at +20, –15, and –65°C. After measuring the signal at –15°C, the sample temperature was increased to +20°C and then decreased to –65°C. The redox potential of the reaction medium was +260 mV. Upward and downward arrows show the moments of actinic light on and off, respectively.

RESULTS AND DISCUSSION

It is most probable that at room temperature and steady-state photoactivation, photooxidized cytochrome *c* is reduced by electron transfer from the exogenous donor (DCPIP reduced with sodium ascorbate). This reaction is obviously controlled by diffusion. Upon decreasing the temperature to 240-250 K, the rate of dark reduction of photooxidized cytochrome *c* significantly decreased (Fig. 1). At a temperature of about 250 K, the photooxidation of cytochrome *c* became virtually irreversible. The Arrhenius plot of the reaction rate constant within the temperature range from 300 to 260 K is virtually a linear function with the activation energy E_a = 9.9 kcal/mol (Fig. 2). It should be noted that this value is close to the activation energy of thermal diffusion. The dependence shown in Fig. 2 was plotted for the slow component of absorption changes, which corresponded to the dark reduction of cytochrome *c*. The half-time of the process increased from 25 sec at 293 K to 180 sec at 263 K. It should also be noted that approximately the same activation energy was typical of the fast component of the process (2 and 15 sec at 293 and 263 K, respectively) (not shown in Fig. 2).

However, the pattern of photoinduced reactions of cytochrome *c* changed considerably upon further temperature decrease to 150-220 K. The photoinduced oxidation of cytochrome *c* within this temperature range was virtually completely reversible. The amplitude of the photoinduced signal declined with the temperature decrease, whereas the kinetics of the dark reduction of photooxi-

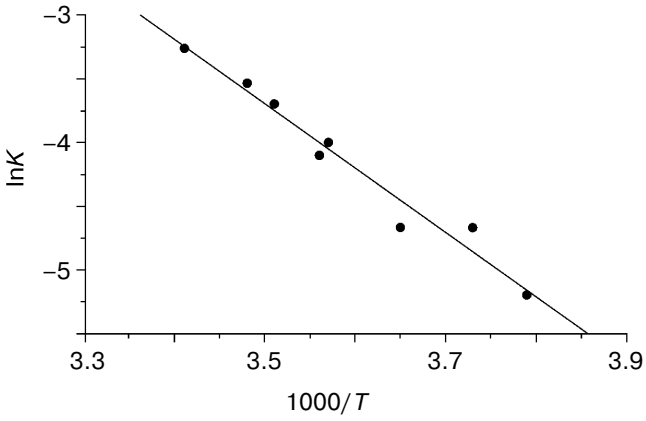

Fig. 2. Arrhenius plot of the reaction rate constant of dark reduction of cytochrome *c* within the temperature range from 300 to 260 K in RC complexes of *Rps. viridis*. The dependence is approximated by a linear function with activation energy E_a = 9.9 kcal/mol.

dized cytochrome was biphasic and virtually independent of temperature (characteristic times of fast and slow kinetic components were 5-8 and 70-100 sec, respectively). Although reversible photooxidation of cytochrome *c* was observed in *Chromatium* chromatophores by Dutton and Prince [10] more than 20 years ago, detailed kinetic analysis of this phenomenon has not been performed so far. It is obvious that isolated reaction centers are more appropriate objects for such analysis than chromatophores.

Consider possible mechanisms of dark reduction of photooxidized cytochrome *c* in the *Rps. viridis* RC complexes at low temperature. Because this reaction is virtually completely reversible and can be reproduced many times at cryogenic temperatures, when thermal diffusion is inhibited, it is reasonable to suggest that oxidized cytochrome *c* receives electrons as a result of reversion of the light-induced reactions. A similar mechanism was considered in [10]. Direct electron tunneling from the photoreduced quinone acceptor to photooxidized cytochrome *c* seems to be rather improbable because the distance between these carriers is too large. Indeed, according to the X-ray diffraction data, this distance is about 4.8 nm: ~2.8 nm between the primary quinone Q_A (menaquinone) and bacteriochlorophyll dimer plus ~2.0 nm between the bacteriochlorophyll dimer and the nearest cytochrome *c* heme (distances between centers of macrocycles) [1]. Consider the scheme of electron transport pathways in the *Rps. viridis* RC in more detail. Because at $T < 240$ K the reactions of electron transfer from exogenous donor to cytochrome *c* and from the primary quinone acceptor to the secondary quinone acceptor are blocked [9], the following scheme of dark reactions is suggested:

$$C \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} RC \overset{k_2}{\leftarrow} Q_A \ .$$

The equilibrium constant $K = k_1/k_{-1}$ can be calculated from free energy changes (ΔG) of this reaction:

$$\Delta G = -RT \ln K, \qquad (1)$$

where R is the universal gas constant; T is temperature.

Therefore, the characteristic time of the dark reduction of the cytochrome can be calculated as:

$$\tau = 1/k_{-1} = K/k_2 = K\tau_2 = \tau_2 \exp(-\Delta G/RT) \ , \qquad (2)$$

where τ_2 is the characteristic time of recombination between photooxidized bacteriochlorophyll dimer and photoreduced primary quinone acceptor Q_A.

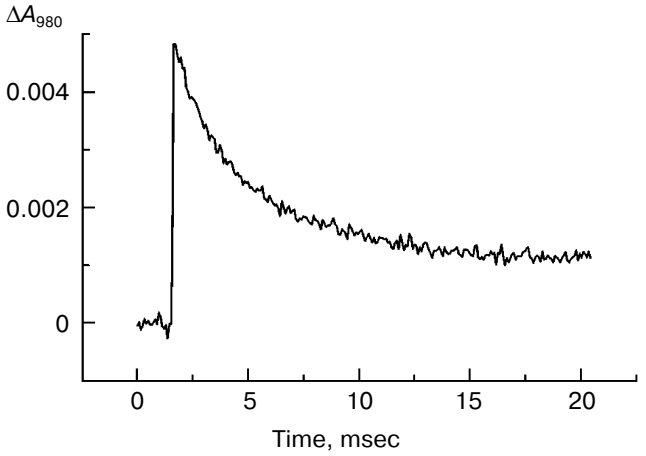

Fig. 3. Light-induced absorption changes at 980 nm in RC complexes of *Rps. viridis* in the presence of 10 mM *o*-phenanthroline, an inhibitor of electron transfer from the primary to the secondary quinone acceptors. Photoactivation with an ISSh-100 3M flash lamp (pulse duration, 10 μsec; spectral range, 400-600 nm; pulse energy, 9 mJ).

The value of τ_2 in the RC preparations studied in this work was measured experimentally under oxidative conditions in which cytochrome *c* was chemically oxidized and in the presence of *o*-phenanthroline, an inhibitor of electron transfer from the primary to the secondary quinone acceptor (Fig. 3).

The value of ΔG can be assessed from the standard redox potentials of the corresponding electron carriers.

The four-heme cytochrome *c* of the *Rps. viridis* RC contains two high-potential hemes that can donate electrons to photooxidized bacteriochlorophyll dimer under the experimental conditions studied in this work (under aerobic conditions low-potential hemes are oxidized by air oxygen). The values of mid-point redox potentials (E_0') of high-potential hemes are +310 and +380 mV [2] (c_{310} and c_{380}, respectively). According to the results reported by different researchers, the value of E_0' of the RC bacteriochlorophyll dimer in *Rps. viridis* ranges from +500 to +520 mV [2, 11]. Thus, the energy gap between bacteriochlorophyll dimer and high-potential cytochrome hemes is 190-210 and 120-140 mV. Substitution of these values and value of τ_2 in Eq. (2) gives the values of τ equal to 1-3 and 50-150 sec. To a first approximation, these values correspond to the characteristic times of the fast and slow components of the dark reduction of photooxidized cytochrome *c* measured in our experiments. Therefore, the recombination mechanism of dark reduction of photooxidized cytochrome *c* is thought to be consistent with experimentally measured values of kinetic and thermodynamic characteristics of RC components.

Although the values of E_0' and τ_2 of the RC electron carriers were measured at room temperature, whereas

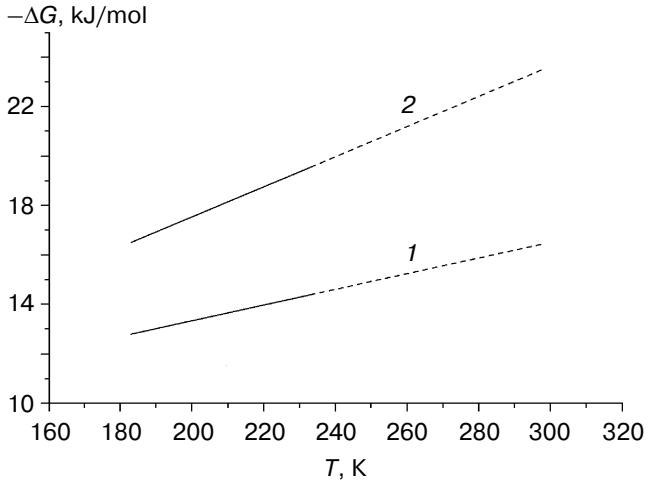

Fig. 4. Temperature dependence of the free energy changes of electron transfer from cytochrome *c* to photooxidized bacteriochlorophyll dimer in RC complexes of *Rps. viridis* as calculated from Eq. (3) for hemes c_{380} (*1*) and c_{310} (*2*), respectively. Dotted lines show approximation to room temperature.

kinetic characteristics of electron transport reactions were measured at low temperatures, the approximation considered above seems to be valid. Indeed, it was shown in [12] that the characteristic time of recombination between photooxidized bacteriochlorophyll dimer and photoreduced primary quinone acceptor is virtually independent of temperature. On the other hand, the literature contains very little information on the temperature dependence of the E_0' values of the bacterial RC components. In particular, redox titration of high-potential hemes of cytochrome *c* and special pair of bacteriochlorophyll molecules was performed in *Chromatium vinosum* chromatophores within the temperature range from 300 to 280 K [13]. Although it was shown in these experiments that upon decreasing the temperature, the values of E_0' of both of high-potential hemes of cytochrome *c* and the special pair of bacteriochlorophyll molecules were shifted, the energy gap between the electron levels of these carriers was changed only insignificantly [13]. Therefore, the extrapolation used in our calculations seems to be appropriate. Moreover, this approach can be used to solve the inverse problem of estimation of the temperature dependence of changes in the free energy of the reaction (therefore, temperature dependence of the E_0' values of electron carriers) based on kinetic measurements:

$$\Delta G = RT \ln(\tau_2 / \tau) . \quad (3)$$

The results of corresponding calculations for two high-potential hemes of the *Rps. viridis* cytochrome *c* (c_{380} and c_{310}) are shown in Fig. 4. Linear approximation of these results to room temperature gives the values of ΔG equal to 16.5 and 23.6 kJ/mol, respectively. These values are close to the free energy changes calculated from the curves of redox titration of corresponding carriers.

On the basis of these temperature dependencies, we calculated changes in enthalpy (ΔH) and entropy (ΔS) of the reaction of electron transfer from the cytochrome hemes c_{310} and c_{380} to bacteriochlorophyll dimer in the *Rps. viridis* RC:

$$\Delta G = \Delta H - T \Delta S . \quad (4)$$

Changes of enthalpy and entropy for heme c_{380} were estimated as −6.87 kJ/mol and 32.3 J/(mol·K). These values for heme c_{310} were estimated as −5.24 kJ/mol and 61.6 J/(mol·K), respectively. The results of our calculations are sufficiently close to the values obtained by direct redox titration in chromatophores of *Chromatium vinosum* (ΔH = −8.8 kJ/mol and ΔS = 19.3 J/(mol·K)) [13] and chromatophores of *Ectothiorhodospira shaposhnikovii* (ΔH = −1.67 kJ/mol and ΔS = 26.8 J/(mol·K)) [14]. Thus, the method of estimation of thermodynamic characteristics of the reaction of electron transfer from cytochrome *c* to photooxidized RC bacteriochlorophyll dimer suggested in this work is in conformity with the results of other methods. However, in contrast to the method of direct redox titration, the method suggested in this work can be used within a significantly wider range of temperature.

Thus, the recombination mechanism considered in this work not only provides a consistent description of the kinetics of dark reduction of photooxidized cytochrome *c* at low temperature but also allows the thermodynamic characteristics of corresponding transitions to be estimated.

This study was supported by the Russian Foundation for Basic Research (project No. 00-04-48106) and International Scientific-Technological Center (project No. 866).

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