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EVIDENCE FOR THREE PHOTOCHEMICAL SYSTEMS IN *CHROMATIUM* D

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SUMMARY

1. The action spectra for oxidation of cytochrome c_{553}, cytochrome *cc'* and cytochrome c_{555}* in *Chromatium* D were studied.

2. Light intensity dependency of the photoinduced oxidation of the hemoproteins was studied, using light having wavelengths of 813, 853 and 904 nm as actinic light.

3. In summing up the results of the experiments, 810 nm light was more favorable for the oxidation of cytochrome c_{553}; 850 nm light was more effective for the oxidation of cytochrome *cc'*; and cytochrome c_{555} was oxidized more effectively by 890 nm actinic light.

4. On the basis of these findings, the existence of three photochemical reaction systems in *Chromatium* was hypothesized.

INTRODUCTION

Since its discovery by DUYSENS[1] in 1954, the photoinduced oxidation of cytochromes in the photosynthetic bacteria has been extensively studied. In *Chromatium* it has been shown that the photoinduced oxidation of the three hemoproteins, cytochrome c_{553}, cytochrome c_{555} and cytochrome *cc'* is represented by three distinct paths of electrons[2–4]. These three reactions were found to differ from each other in several respects; reactivity at low temperatures[5], kinetics of dark reduction of the oxidized forms[2,4], dependency on light intensity[2], sensitivity towards various inhibitors[4], sensitivity to oxygen[2,4] as well as in the effects upon them of endogenous or added substrates[4].

On the other hand, bacteriochlorophyll of *Chromatium* occurs in the cell in three major forms distinguished by the absorption bands in the near-infrared region, at 810, 850 and 890 nm, with minor components[6,7]. AMESZ AND VREDENBERG[8] investigated the relationship between the photoinduced oxidation of the hemoproteins and the

* The components of the cytochrome systems in *Chromatium* were named according to the suggestions of BARTSCH[13] and the Recommendations (1964) of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes[14]. Cytochrome c_{553}: cytochrome $c_{423.5}$, lower reduction–oxidation potential *c*-type cytochrome. Cytochrome *cc'*: cytochrome c_{426}, cytochromoid *c*, RHP. Cytochrome c_{555}: cytochrome c_{422}, high-potential *c*-type cytochrome, cytochrome c_2-like cytochrome.

forms of bacteriochlorophyll, by measuring the action spectrum for one of these reactions, *i.e.*, oxidation of cytochrome c_{553}.

In the present study the photoinduced oxidations of these three hemoproteins were investigated in an attempt to correlate each reaction with one particular form of bacteriochlorophyll. The experiments were carried out in two ways. The action spectra for the oxidation of the three hemoproteins were measured separately under various specific experimental conditions, using intact cells of *Chromatium*. Relative efficiencies of actinic light at various wavelengths were also compared with respect to the steady state of oxidation of the hemoproteins.

MATERIAL AND METHODS

Chromatium D was cultured as described in the previous paper[4]. The 'starved cells', prepared as described in the previous paper, were used throughout this study[4]. The substrate, or hydrogen donor, was the culture solution (containing thiosulfate, sulfite and CO_2) used as suspending medium.

The absorbance changes were measured in an Aminco–Chance dual-wavelength spectrophotometer, with a modified sample compartment. A 3-mm lightpath cuvette was mounted at the end of the sample compartment adjacent to the detector. The actinic light was furnished from a Bausch and Lomb monochromator through an 88A (Wratten) filter. The sample cuvette was illuminated from the same side but at an angle of 15° to the measuring beam. A blue-colored filter VB 46 (Toshiba) was inserted as a complementary filter between the sample and the photomultiplier of the spectrophotometer.

The intensity of the actinic light was measured with a Si-photocell (Solar Systems, Inc., SSII L) calibrated with a thermopile (Kipp and Zonen, Type E1) and a standard incandescent lamp (National Bureau of Standards). For the measurement of the light intensity, the Si-cell was placed in the position of the sample cuvette in the spectrophotometer. The intensity of actinic light was changed by insertion of a calibrated neutral density filter (Hoya, Glass Works, Ltd.).

In some experiments, monochromatic light employed as the actinic light was obtained by using the combination of an interference filter (Tokyo Denshi Kogaku) and a color filter (Wratten 88A) and the intensity of actinic light was changed by adjusting the input voltage of the lamp used as the light source.

The absorption spectrum was measured in a Cary spectrophotometer Type 14 and Shimazu MPS-50 spectrophotometer. For correction of the light-scattering effect of the sample, the absorbance was calibrated at 810, 850 and 890 nm by means of the integrating sphere method.

RESULTS

Action spectra

The photoinduced oxidation of the individual hemoproteins, cytochrome c_{553}, cytochrome c_{555} and cytochrome *cc'* in *Chromatium* cell was measured separately at various wavelengths of actinic light. The light intensity inducing a given amount of change in the steady-state level of oxidation of each hemoprotein was obtained by interpolating or extrapolating the light intensity curve for each wavelength of actinic

light. The action spectrum for each hemoprotein was drawn by plotting the reciprocals of the values for light intensity thus obtained (Fig. 1).

The photoinduced oxidation of the hemoprotein was obtained separately in the following ways:

(i) Under anaerobic conditions and in the presence of the substrate in the reaction medium, illumination at lower intensities of actinic light causes oxidation of cytochrome c_{553}, without inducing any change in steady-state level of other hemoproteins in the bacterial cell[2,4].

(ii) On illumination under aerobic conditions, and without addition of the substrate, the starved cells only show a change in absorbance corresponding to the oxidation of cytochrome c_{555} (refs. 2, 4).

(iii) In the presence of a high concentration of 1,10-phenanthroline (10^{-3} M), the photoinduced oxidations of cytochrome c_{553} and cytochrome c_{555} were eliminated and on illumination at higher intensities of actinic light, the photoinduced oxidation of cytochrome *cc'* alone was observed[4]. A small amount (0.05 ml) of ethanol solution of 1,10-phenanthroline was applied to the cell suspension about 15 min prior to the measurement. The reaction mixture was kept in the light at room temperature to produce the full effect of the inhibitor.

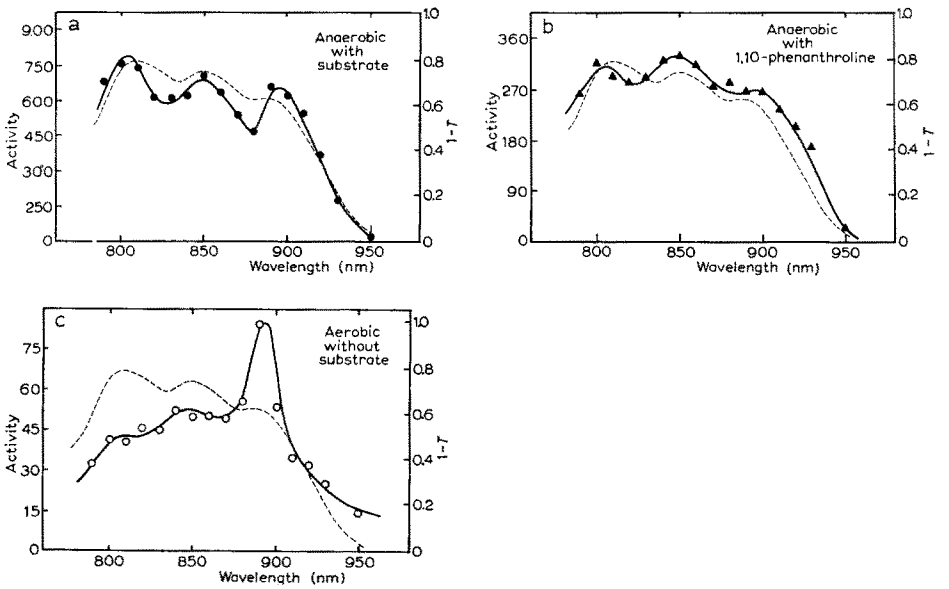

Fig. 1. a. Action spectrum for photoinduced oxidation of cytochrome c_{553}. Activities are expressed by the reciprocals of flux of quanta (10^8 Einstein^{-1}·sec·cm^2), inducing 0.8% change in $\Delta T_{423.5-440\ nm}$. Anaerobic, with substrate. Half-width of actinic light was 5 nm. Amounts of bacterial cells used, 1.86 mg Folin protein/ml. Dashed curve, absorption spectrum. b. Action spectrum for photoinduced oxidation of cytochrome *cc'*. Activities are expressed by the reciprocals of flux of quanta (10^8 Einstein^{-1}·sec·cm^2), inducing 1.0% change in $\Delta T_{423.5-440\ nm}$. Anaerobic, with substrate. 10^{-3} M 1,10-phenanthroline had been added 15 min prior to measurement. Half-width of actinic light was 10 nm. Amounts of bacterial cells used, 1.86 mg Folin protein/ml. Dashed curve, absorption spectrum. c. Action spectrum for photoinduced oxidation of cytochrome c_{555}. Activities are expressed by the reciprocals of flux of quanta (10^8 Einstein^{-1}·sec·cm^2), inducing 0.15% change in $\Delta T_{423.5-440\ nm}$. Aerobic, without substrate. Half-width of actinic light was 20 nm. Amounts of bacterial cells used, 1.86 mg Folin protein/ml. Dashed curve, absorption spectrum.

The resulting action spectra are shown in Figs. 1a, b and c. The action spectra approximately follow the absorption spectrum of the intact cells showing three peaks in the near-infrared region. On closer examination, however, meaningful differences were discovered with respect to the precise shapes of the spectral curves.

A striking difference will be seen in relative efficiencies at 810 and 890 nm. 810 nm light is more effective for oxidation of cytochrome c_{553}, while for the oxidation of cytochrome c_{555}, 890 nm light is more efficient. In the oxidation of cytochrome *cc'*, the discrepancy between the action and the absorption spectra is not very marked. However, it may be stated that 850 nm light is most effective for the oxidation of cytochrome *cc'*, as compared with the light of the other two absorption bands of bacteriochlorophyll (Fig. 1b). The precise shape of the action spectra for each reaction differed slightly from bacterial sample to sample, depending upon the culture conditions and other unidentified factors. But such differences, if any, are too small by far to invalidate the correlation between kinds of cytochrome reactions and forms of bacteriochlorophyll.

These facts lead us to the inference that the reaction systems for the observed oxidations of cytochrome c_{553}, cytochrome *cc'* and cytochrome c_{555} are preferentially closely connected to the three forms of bacteriochlorophyll, 810, 850, and 890 nm forms, respectively.

Actinic light intensity vs. steady-state level of photoinduced oxidation

In the experiments described in the previous section, the oxidations of the hemoproteins were run under significantly different reaction conditions. There is a possibility that some of the conditions might have affected the energy transfer within the bacteriochlorophyll system. Another set of experiments was therefore designed to confirm the connection between the hemoprotein oxidations and the three forms of bacteriochlorophyll. The photoinduced oxidations of the hemoproteins were measured by following the changes in steady-state level of absorbance at 423.5 nm (reference wavelength: 440 nm). This wavelength corresponds to the maximum of the light-*minus*-dark difference spectrum for cytochrome c_{553}; this is also in the proximity of the corresponding maxima for the other hemoproteins. 90 and 80% of the maximal changes to be obtained on the oxidation of cytochrome c_{555} and cytochrome *cc'*, respectively, were observed at this wavelength of measurement. The observed changes were plotted against intensities of actinic light (Fig. 2).

By comparison of the curves in Fig. 2, it will be seen that the reaction which

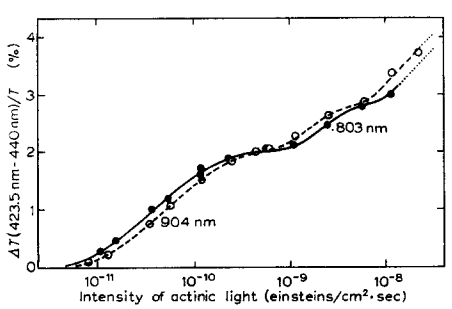

Fig. 2. Relationship between amplitudes of absorption changes and actinic light intensities. Anaerobic, with substrate. Dashed curve, 904 nm light. Solid curve, 803 nm light.

is saturated at lowest range of light intensity is more effectively induced by 803 nm light than by 904 nm light. For 50% saturation of the low intensity reaction, the required intensity of 803 nm light is 25% lower than the required intensity of 904 nm light. This step of light-induced reaction corresponds to the oxidation of cytochrome c_{553} (refs. 2, 4).

The second step in the light intensity curves which reflects the oxidation of cytochrome *cc'* was subject to significant fluctuation. Therefore, a detailed study of this step of reaction was not made.

The third step, oxidation of cytochrome c_{555}, responds preferentially to the light of 904 nm than to light of other wavelengths. The inflection point for the third step of the reaction was estimated from the results of another series of experiments, covering higher ranges of light intensities.

These results are summarized in Table I in terms of the light intensities corresponding to the inflection point of each step of oxidation of hemoproteins.

TABLE I

ACTINIC LIGHT INTENSITIES CORRESPONDING TO THE INFLECTION POINTS IN THE LIGHT INTENSITY CURVES OF PHOTOINDUCED OXIDATION OF HEMOPROTEINS (ABSORPTION CHANGES AT 423.5 nm)

Expt. 1: the same as in Fig. 2; Expt. 2: anaerobic, with substrate; amounts of bacterial cells used, 2.5 mg Folin protein/ml.

Expt. No.	*Reactions**	*Hemoproteins*	*Intensity of inflection point (Einstein·cm^{-2}·sec^{-1})*		
			803 nm	*853 nm*	*904 nm*
1	I	cyt c_{553}	$4.1 \cdot 10^{-11}$	$4.4 \cdot 10^{-11}$	$5.5 \cdot 10^{-11}$
2	I	cyt c_{553}	$9.8 \cdot 10^{-11}$	$9.8 \cdot 10^{-11}$	$13.0 \cdot 10^{-11}$
	III	cyt c_{555}	$1.4 \cdot 10^{-8}$	$1.1 \cdot 10^{-8}$	$1.0 \cdot 10^{-8}$

* Data for Reaction II are omitted (see text).

The intensities of the inflection points under investigation are quantities which depend on, among other factors, the concentration of the bacterial cells used in the experiment. In fact, the actual ratios of the above-defined intensity for the three wavelengths of actinic light were also subject to fluctuation, especially with respect to the second-step reaction. For this reason, the values for the second step are not indicated in the table. Nevertheless, the respective order in intensities of the inflection points for each step of the reaction remained unaltered, so far as the first and the third steps were concerned.

In summary, it may be concluded from the experimental results that the photo-induced oxidation of cytochrome c_{553} is most effectively promoted by 803 nm light, and that of cytochrome c_{555} by the 904 nm light. These results conform with the data of the action spectra for the oxidations of the individual hemoproteins.

DISCUSSION

The fact that the action spectra for the oxidations of the hemoproteins obtained under various conditions clearly differ from each other, as well as from the absorption

spectrum of the intact cells, suggests a possible differentiation in function of the three forms of bacteriochlorophyll, 810, 850 and 890 nm forms, in *Chromatium*. The results of another series of experiments, in which the effects of light intensity on the steady-state level of oxidation were compared at various wavelengths, led to the same conclusion. The observed differences in shape of action spectra obtained under various conditions were not artifacts due to differences in experimental conditions under which the reaction was measured.

Evidence has been obtained showing that separate electron transporting paths are operating in the photoinduced oxidation of the hemoproteins in *Chromatium*[4,5,9]. The results of this study indicate that each one of the three electron transport reactions is correlated to one of the three forms of bacteriochlorophyll, or it may be that each of these reactions is correlated to one bacteriochlorophyll system in which one of the three forms of bacteriochlorophyll is dominant, to form three distinct photochemical reaction systems in the photosynthetic mechanism of *Chromatium*. The interrelationship of these reaction systems, however, is still obscure.

Possibilities of two kinds of photoreaction in photosynthetic bacteria have been suggested by CLAYTON[10] and VREDENBERG and co-workers[6,11], based on the finding of two kinds of bleaching of bacteriochlorophyll-like pigments supposed to be the reaction center of photochemical reactions.

AMESZ AND VREDENBERG[8] suggested the compartmentation of light-harvesting pigments in photosynthetic bacteria, on the basis of the findings concerning the quantum yields of fluorescence and other photochemical reactions, the oxidation of cytochrome and the shift of absorption bands of carotenoids. They have measured the oxidation of only one component of the hemoproteins in the bacterium (*Chromatium*), *i.e.*, cytochrome c_{553}. More direct evidence for the presence of three different photoreaction systems in photosynthetic bacterium is brought forth in the present study by comparing the photoinduced oxidations of the three hemoprotein components in *Chromatium* cells.

GARCIA and co-workers[12] reported a separation of two kinds of pigmented particles in *Chromatium* by means of degradation of the chromatophores with Triton X-100, followed by separation by density-gradient centrifugation. The two kinds of fractions thus obtained showed different ratios of the bacteriochlorophyll forms. Their results are of interest in connection with the possible compartmentation of bacteriochlorophyll as suggested by the results of the present work.

It has to be noted in this connection that there has been no indication of any 'red drop' in the action spectra of photoinduced oxidation of the hemoproteins in *Chromatium*. Nor was any single-peaked action spectrum obtained for the hemoprotein oxidation. These facts indicate that there is a strong interaction among the photochemical systems in question. Various alternatives are possible as a mechanism for such interaction.

(i) There may be a separation of the electron transport systems into three compartments, in each one of which all three bacteriochlorophyll forms are involved, but in different amounts.

(ii) The pigment systems may have a strong connection with each other, so that transfers of excited energy take place among the three pigment systems.

(iii) The interaction may occur in the steps of electron transport.

A combination of these possible interactions is also possible. In any case, the

experimental results indicate that the separation of the reaction systems suggested seems to be a rather loose one.

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