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THERMODYNAMICS OF THE PRIMARY AND SECONDARY PHOTOCHEMICAL REACTIONS IN *CHROMATIUM*

GEORGE D. CASE AND WILLIAM W. PARSON
Department of Biochemistry, University of Washington, Seattle, Wash. 98195 (U.S.A.)

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SUMMARY

Thermodynamic properties of the primary and secondary photosynthetic electron transfer reactions in *Chromatium vinosum* have been studied with the aid of short actinic light flashes. Midpoint redox potentials measured for the primary electron acceptor (X), P870, and cytochromes C555 and C552 agreed with those which previous workers have obtained with other techniques.

The secondary electron acceptor (Y) has a midpoint redox potential at pH 7.7 ($E_{m7.7}$) of approx. −90 mV, and is present in a pool of approx. two molecules per molecule of X. The reaction between X^- ($E_{m7.7}$ approx. −130 mV) and Y is a single-electron process, which occurs at the same rate whether the Y pool is completely oxidized or partially reduced.

The possibility was considered that the E_m values might be sensitive to electrostatic interactions between electron carriers within a photosynthetic unit. However, the redox titrations of cytochrome C555 appear to be unaffected by the oxidation state of X. Similarly, oxidation of one of the two cytochrome C555 hemes appears not to perturb the titration of the other heme. As a result, values of $\Delta G°$, $\Delta H°$, and $\Delta S°$ derived from redox titrations of the electron carriers in separate photosynthetic units probably represent the true standard state values for the photochemical reactions.

Measurements of the E_m values as functions of temperature allowed a resolution of the free energy changes ($\Delta G°$) into the entropy and enthalpy changes ($\Delta S°$ and $\Delta H°$), for each of the reactions. Surprisingly, an entropy decrease accounts for all of the free energy which is stored in the primary photochemical reaction. Overall values of $\Delta G°$, $\Delta H°$, and $\Delta S°$ for the transfer of an electron from cytochrome C555 to Y are +9.9 kcal/mole, −0.7 kcal/mole, and $-35.9\,\mathrm{cal}\cdot{}^{\circ}\mathrm{K}^{-1}\cdot\mathrm{mole}^{-1}$, respectively.

The E_m values of the cytochromes are independent of pH. Those of P870, X, and Y all depend on pH, indicating the uptake of approx. $\frac{1}{2}H^+$ in each of the half-cell reductions.

N-Methylphenazonium methosulfate (PMS), at a concentration of 100 μM, does not alter the redox titrations of P870, X, or either cytochrome C555 or cytochrome C552. However, it does interfere with titrations of Y, raising the apparent midpoint potential and increasing the pH dependence of the titration, in a manner which does not directly correlate with the chemical reduction of PMS itself.

Abbreviation: PMS, *N*-methylphenazonium methosulfate.

INTRODUCTION

Previous work by several authors[1-9] gives a basic picture of the energetics of the primary electron transport reactions in bacterial photosynthesis. The midpoint redox potentials of the primary electron donor (a special bacteriochlorophyll complex called P870[1]) and the primary electron acceptor (called "X") have been studied in several species of photosynthetic bacteria. Values ranging from +400 to +500 mV for P870, and 0 to −200 mV for X, have been measured in *Chromatium*[3-6], *Rhodopseudomonas spheroides*[6,7], and *Rhodospirillum rubrum*[6,8,9].

In addition, *c*-type cytochromes have been implicated as the secondary electron donors in *Chromatium*[1,10,11] and in *Rhodopseudomonas viridis*[12,13]. Present in *Chromatium* are a high-potential cytochrome (C555) with E_m approx. +330 mV[3,14], and a low potential cytochrome (C552) with E_m approx. +10 mV[3,4,13]. Either cytochrome can donate electrons to the same reaction center bacteriochlorophyll, P870, under the proper conditions[13,15]. In *Chromatium*, there appear to be two cytochrome C555 hemes per P870 complex[16].

Reduced X (X^-) transfers an electron to a secondary acceptor, called "Y"[17]. The kinetics of this reaction have been studied in *Chromatium*[13,15,17], but the thermodynamics and pool sizes have not been discussed previously.

The purpose of the present work is to explore in greater depth the energetics of the primary and secondary photochemical reactions in *Chromatium*. This report describes attempts to establish the pool size and midpoint redox potential of Y, determinations of the standard state enthalpy and entropy changes in each of the primary and secondary reactions, and a study of the influence of the pH on the reactions. The use of short actinic flashes has allowed a more decisive resolution of some of the electron carriers than was available to previous workers, who used steady-state techniques.

EXPERIMENTAL

Chromatium vinosum (*Chromatium* strain D) was grown photoautotrophically either in the medium of MORITA *et al.*[18] or in the medium whose composition is shown in Table I. Cells were grown for a period of about 2.5 days under continuous illumination from either tungsten or infrared lamps. We found the medium of Table I advantageous because, unlike the medium of MORITA *et al.*[18], it consists of odorless,

TABLE I

Chromatium GROWTH MEDIUM

Solutions A, B, and C are combined in the ratio 130:130:1, by vol., just prior to inoculation.

Solution A		*Solution B*		*Solution C*	
NaCl	60 g	$Na_2S_2O_3 \cdot 5H_2O$	6 g	$FeSO_4 \cdot 7H_2O$	1.6 g
K_2HPO_4	1 g	$NaHCO_3$	8 g	EDTA	3.0 g
KH_2PO_4	1 g	Water	1 l	Water	1 l
NH_4Cl	2 g			Adjust pH to 7.5	
$MgCl_2 \cdot 6H_2O$	1 g				
$CaCl_2$	0.2 g				
Water	1 l				

clear solutions which are stable indefinitely. Preparation of chromatophores followed standard procedures[19].

The spectrophotometric methods for measuring oxidation and reduction of the cytochromes and P870, after a single laser flash or a pair of laser flashes of saturating intensity, have been described elsewhere[17]. In some experiments, the signal from the photomultiplier was digitized and stored in the memory of a Biomation model 610 transient recorder. A third actinic flash was provided for some experiments by a Xe flash lamp and a Corning 2600 filter. The pulse width was 5 μsec at half-maximal amplitude. The actinic effect of the Xe flash for cytochrome oxidation was about 85 percent of maximal.

For measurements of BChl fluorescence, a Xe lamp provided an excitation flash with a pulse width of 8 μsec at half-maximal amplitude. Blue-green filters (Corning 9782, and Schott BG-18, GG-4, and GG-10) were used in the excitation path, and near-infrared filters (Corning 2540 and 2600, and Schott RG-9) in the fluorescence detection path, which was normal to the excitation path. The output signal from the photomultiplier was fed through a pulse amplifier (C-Cor 4388F) to an oscilloscope. The fluorescence excitation flash was attenuated with neutral density filters until its actinic effect for cytochrome oxidation was less than one percent of maximal. Several minutes elapsed between flashes.

The methods for controlling and measuring the redox potential and the temperature have been described previously[15, 17]. Unless otherwise indicated, the temperature was 22°. The calomel reference electrode was calibrated by measuring the redox potential of a saturated quinhydrone solution as a function of pH at five different temperatures[20]. This technique gave $E = +240$ mV for the calomel electrode at 22°. The electrode calibration obtained from redox titrations of methylene blue and flavin mononucleotide gave a value of $E = +225$ mV for the same electrode. The reason for the 15 mV discrepancy between these two calibrations is not clear. We have used the quinhydrone calibration for all of the E_m values which are presented below. The possibility of a small systematic error in the absolute E_m values should not propagate to the calculations of ΔG°, ΔH°, and ΔS° for the electron transfer reactions, as the latter quantities arise from differences between E_m values. All redox potentials are given relative to the standard hydrogen electrode (E_h, according to CLARK[20]). Definitions of E_m, E_{m7}, and standard E_{m7} values were taken from CLARK[20]. All of the redox titrations were reversible, if exposure to actinic light was minimized.

The following redox buffer systems were used in most of the cytochrome titrations. Buffer A: 50 μM ferric EDTA, 100 μM potassium indigotetrasulfonate, and 100 μM potassium indigodisulfonate. Buffer B: 100 μM *N*-methylphenazonium methosulfate (PMS), 100 μM indigotetrasulfate, and 100 μM indigodisulfate. Other redox buffers were used in some cases as indicated below. Most of the titrations were done in the presence of 0.1 M potassium phosphate. For the titrations in which the pH was below 6 or above 8, 0.1 M Tris–maleate was used instead.

In P870 titrations, $K_3Fe(CN)_6$ was used to oxidize endogenous reductant, and it served as the sole redox buffer. Prior to the titration, the chromatophores were either dialyzed for 6 h against 1 mM $K_3Fe(CN)_6$ or treated with aliquots of 0.1 M $K_3Fe(CN)_6$ to oxidize all of the P870. Reductive titrations then were carried out with $Na_2S_2O_4$.

In the steady-state titrations of cytochrome C555 (Fig. 4B), no actinic flash was used, and the oxidation state of the cytochrome was measured continuously on an Aminco-Chance dual-wavelength spectrophotometer. The measuring wavelength was 422 nm; the reference, 436 nm. Enough $K_3Fe(CN)_6$ was added to raise the ambient redox potential above +420 mV, and reductive titrations were carried out with $Na_2S_2O_4$. Either PMS (100 μM) or dichlorophenolindophenol (100 μM) served as an additional redox buffer in these titrations.

The continuous background illumination which was used in some of the steady-state titrations was supplied by a tungsten-iodine lamp with a H_2O heat filter and two Corning 2600 filters. The irradiance of the continuous light was about 10^{-7} einstein·cm^{-2}·sec^{-1}, which was sufficient to oxidize 75% of the cytochrome C555.

In the steady-state titrations of cytochrome C552 (Fig. 5B), the oxidation state of the cytochrome was measured continuously on the dual-wavelength spectrophotometer, in the absence of actinic illumination. The measuring wavelength was 423 nm; the reference, 437 nm. Enough solid $Na_2S_2O_4$ was added initially to lower the ambient redox potential below −100 mV, and oxidative titrations were carried out with air.

Titrations of PMS (Fig. 2C) were followed spectrophotometrically at 387 nm with a monochromator band pass of 3.0 nm. $Na_2S_2O_4$ served as the reductant.

RESULTS

P870

Fig. 1 presents a typical redox titration of the P870 oxidation which results from two actinic flashes. As one lowers the redox potential, reducing P870$^+$, P870 photooxidation first increases, with $E_{m7.7}$ = + 490 mV (Fig. 1). As the redox potential is lowered further, reducing cytochrome C555 (see below and ref. 1), P870$^+$ reduction after the flashes becomes rapid, and the slow measurements shown in Fig. 1 again detect no P870$^+$.

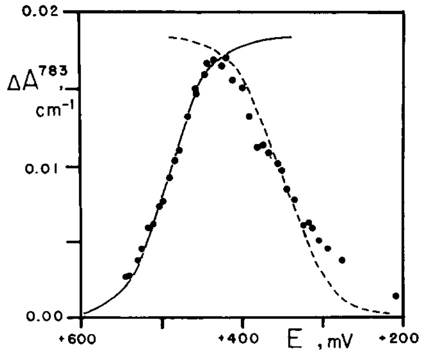

Fig. 1. Redox titration of P870 absorbance changes in *Chromatium* chromatophores. Absorbance measurements at 783 nm after two saturating laser flashes, one msec apart, were followed on an oscilloscope sweeping at 50 msec/cm. Bacteriochlorophyll, 47 μM; pH 7.70. The solid curve represents a theoretical one-electron titration curve with $E_{m7.7}$ = +490 mV; the dashed curve represents a theoretical one-electron titration curve with $E_{m7.7}$ = +348 mV.

Fig. 2A shows that the E_m of the rising limb of the P870 titration decreases with increasing pH, by 23 mV per pH unit. From the data of Fig. 2A, the best estimate of $E_{m7.7}$ for P870 at 22° is +483 mV.

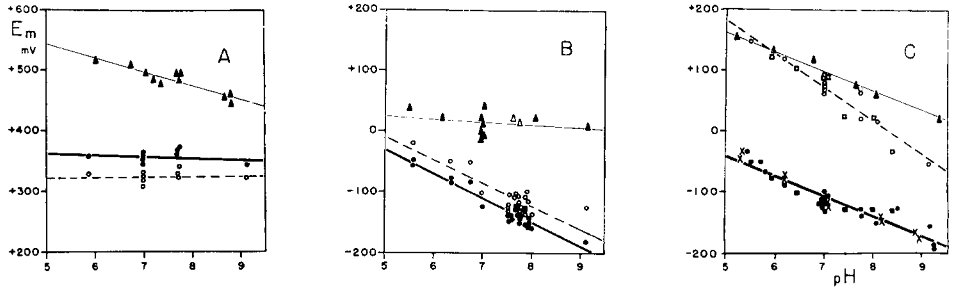

Fig. 2. A. Dependence on pH of the midpoint redox potentials of P870 and cytochrome *c*-555. ▲, E_m values for P870. Conditions as in Fig. 1. The fine solid line was fit to the triangles by linear regression. ●, E_m values for cytochrome C555 obtained from titrations of first flash cytochrome photooxidation. Conditions as in Fig. 4A, except that at pH values other than 7.00, cytochrome absorbance changes were followed at 422 nm. The bold solid line was fit to the points by linear regression. ○, E_m values for cytochrome C555 obtained from titrations of second flash cytochrome photooxidation. Conditions same as for solid circles. The dashed line was fit to the points by linear regression. B. Dependence on pH of the midpoint potentials of X, Y, and cytochrome C552. ●, E_m values for X obtained from titrations of first flash cytochrome photooxidation. Bacteriochlorophyll, 47 μM. Redox buffer A. In some titrations, the concentration of ferric EDTA in the redox buffer was 100 μM. Three points, at pH values of 5.60, 6.37, and 7.95, represent E_m values obtained from oxidative titrations in which the redox potential was adjusted with $K_3Fe(CN)_6$. The presence of 6 mM ATP in some titrations had no apparent effect on the E_m. The bold solid line was fitted to the points by linear regression. ○, uncorrected midpoint potentials for the low-potential falloff of second flash cytochrome photooxidation. Conditions same as for solid circles. The dashed line was fit to the points by linear regression. ▲, E_m values for cytochrome crossover titrations. The ratio of the flash-induced absorbance change at 552 nm to that at 556 nm was followed as a function of the redox potential. Redox buffer B. For experiments at pH 7.0, bacteriochlorophyll, 80 μM. Otherwise, bacteriochlorophyll, 58 μM. △, same as solid triangles, except that redox buffer A replaced buffer B. The fine solid line was fit to all of the triangles by linear regression. C. Dependence on pH of the midpoint potentials of X and Y in the presence of PMS. ▲, E_m values of PMS at different values of pH. PMS, 50 μM. The fine solid line was fit to the points by linear regression. ● and ○, same as solid and open circles in Fig. 2B, except that redox buffer B replaced buffer A. ■ and □, same as solid and open circles, respectively, except that 100 μM methylene blue was also present. The effect of methylene blue alone was not investigated. In some experiments, cytochrome photooxidation was followed at 556 nm or 552 nm, instead of 422.5 nm. Some of the solid circles represent E_m values of oxidative titrations. Bacteriochlorophyll, 55 to 80 μM. The bold solid line was fit to the solid circles and squares, and the dashed line to the open circles and squares, by linear regression. ×, E_m values for X obtained from oxidative and reductive titrations of bacteriochlorophyll fluorescence. Bacteriochlorophyll, 58 μM. Redox buffer B *plus* 100 μM 1,4-anthraquinone-2-sulfonate.

The dependence of the P870 redox titrations on temperature is shown in Fig. 3A. Between 0 and 40°, the $E_{m7.7}$ decreases linearly with increasing temperature. At 22°, the data of Fig. 3A give $E_{m7.7} = +494$ mV. Table II presents the values of $\Delta G°$, $\Delta H°$, and $\Delta S°$ for the P870 half-cell reduction calculated from Figs. 2A and 3A, along with corresponding values for other half-cell reactions. (Calculation of these values is straightforward from the relations $\Delta G° = -nFE_m$, and $\Delta G° = \Delta H° - T\Delta S°$, where F is the faraday and n, the number of electron equivalents transferred per mole, is taken as 1.)

Our values of $E_{m7.7}$ for P870 agree with the $E_{m7.5}$ value of +489 mV reported by CUSANOVICH *et al.*[3], and the $E_{m7.8}$ value of +470 mV reported by DUTTON[4].

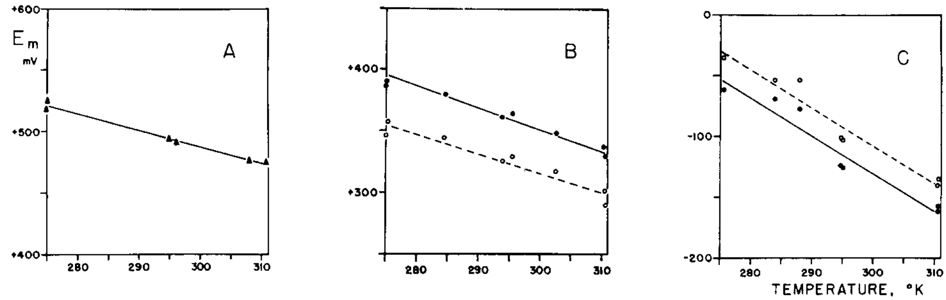

Fig. 3. A. Temperature dependence of the P870 redox titrations. Conditions as in Fig. 1. The solid line was fit to the points by linear regression. B. Temperature dependence of the cytochrome C555 redox titrations. Absorbance changes were measured at 422 nm after the first flash (●) and after the second flash (○). Bacteriochlorophyll, 47 μM. Ferric EDTA, 50 μM; PMS, 100 μM. pH 7.70. Conditions otherwise as in Fig. 4A. Solid line was fit to solid circles, and dashed line to open circles, by linear regression. C. Temperature dependence of the X and Y redox titrations. Conditions as in Fig. 6. ●, E_m values for X. The bold solid line was fit to the points by linear regression. ○, uncorrected midpoint potentials for the low-potential titration of second flash cytochrome photooxidation. The dashed line was fit to the points by linear regression.

TABLE II

STANDARD STATE THERMODYNAMIC VALUES FOR HALF-CELL REDUCTIONS[a] AT 22° AND pH 7.70

Product	$E_{m7.7}$ (*mV*)	$\Delta G°$ (*kcal/mole*)	$\Delta H°$ (*kcal/mole*)	$\Delta S°$ ($cal \cdot °K^{-1} \cdot mole^{-1}$)	H^+ *uptake*[b]	*Footnote*
P870	+494	−11.3	−20.6	−31.5	—	c
	+483	−11.1	—	—	0.40 ± 0.11	d
C555	+341	− 7.9	−18.5	−36.1	—	c, e
	+341	− 7.9	—	—	0.03 ± 0.11	d, e
	+341	− 7.9	—	—	—	f
C552	+ 11	− 0.2	—	—	0.08 ± 0.22	d
	+ 8	− 0.2	—	—	—	g
Y^-	− 83	+ 1.8	−19.5	−72.1	—	c, h
	−101	+ 2.3	—	—	0.65 ± 0.27	d, h
X^-	−116	+ 2.7	−18.5	−71.2	—	c, i
	−134	+ 3.1	—	—	0.63 ± 0.14	d, i
	−131	+ 3.0	—	—	0.62 ± 0.06	d, j

[a] Half-cell reduction with respect to the reaction $\frac{1}{2}H_2 \leftrightharpoons H^+ + e^-$, at unit activities.

[b] Number of protons taken up per electron transferred.

[c] 22° intercept of E_m values in Fig. 3 (all at pH 7.7).

[d] pH 7.7 intercept of E_m values in Fig. 2 (all at 22°).

[e] Intercepts of mean E_m values, taken from titrations of first-flash cytochrome oxidation and second-flash cytochrome oxidation.

[f] Mean E_m value obtained from steady-state titrations similar to Fig. 4B.

[g] Mean $E_{m7.7}$ value obtained from 5 steady-state titrations similar to Fig. 5B, whose E_m values ranged from +2 mV to +15 mV.

[h] A correction of +10 mV has been applied to the E_m values of the low-potential titrations of second-flash cytochrome photooxidation in the absence of PMS.

[i] Obtained from titrations of first-flash cytochrome photooxidation.

[j] Obtained from titrations of bacteriochlorophyll fluorescence.

Cytochromes

Fig. 4A presents typical redox titrations of the cytochrome C555 photooxidation which results from one or two actinic flashes. The titration midpoint for the first flash is about 40 mV more positive than that for the second flash. At potentials above +400 mV, an absorbance increase follows the first flash, but not the second flash. The absorbance increase probably reflects a spectral band of $P870^+$ (ref. 5). Because cytochrome C555 cannot supply an electron to $P870^+$ at potentials above +400 mV, no $P870^+$ reduction occurs in the interim between the two flashes, and no additional P870 is available for photooxidation on the second flash. The absorbance increase due to $P870^+$ changes the high-potential asymptote of the cytochrome titration curve, but it should not affect the shape of the curve or its midpoint potential.

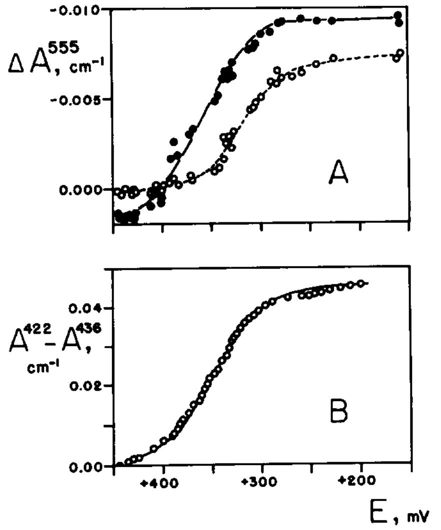

Fig. 4. A. Redox titrations of cytochrome C555. Absorbance changes were measured at 555 nm following the first (●) and second (○) of two saturating laser flashes. Bacteriochlorophyll, 80 μM. PMS, 100 μM. pH 7.0. The solid and dashed curves represent theoretical plots of $2c-c^2$ and c^2, respectively, in which c follows a theoretical one-electron titration with $E_{m7} = +338$ mV (see DISCUSSION). B. Redox titration of cytochrome C555 under steady-state conditions. No actinic illumination. Bacteriochlorophyll, 30 μM. PMS, 100 μM, at pH 7.0. Solid curve represents a theoretical one-electron titration with $E_{m7} = +348$ mV.

Steady-state titrations of cytochrome C555 gave E_m values which were intermediate between the apparent midpoint potentials obtained from the first and second flash measurements (Fig. 4B). The E_m value of +348 mV in Fig. 4B is slightly higher than the mean E_m of +341 mV obtained from 11 similar titrations, whose E_m values ranged from +330 to +360 mV. The steady-state titrations generally gave close fits to the Nernst equation for a single-electron reaction:

$$E = E_m + \frac{RT}{nF} \ln \frac{1-c}{c}$$

where c is the fraction of the cytochrome which is reduced at potential E. Our E_m value of +341 mV is slightly higher than those reported by CUSANOVICH *et al.*[3]

(+319 mV) and OLSON AND OWENS[14] (+330 mV). The presence of gramicidin D (0.6 μg/ml) had no effect on the titrations of cytochrome photooxidation or the steady-state cytochrome titrations.

For some of the steady-state titrations (not shown in the figures), 300 μM 1,10-phenanthroline was added to inhibit electron transfer between X^- and Y[15]; in others, continuous illumination was used to drive electron transfer between P870 and X. In some experiments, both 1,10-phenanthroline and continuous illumination were used. The 1,10-phenanthroline inhibited cytochrome oxidation on the first flash by 10%, and on a second flash by 90%. Either singly or in combination, 1,10-phenanthroline and continuous illumination had no significant effect (less than ±10 mV) on the E_m of cytochrome C555. Thus changes in the redox state of X appeared not to affect the cytochrome titration.

Fig. 3B presents the temperature dependence of the E_m values which were obtained for cytochrome C555 in flash experiments. The E_m values decrease linearly with increasing temperature between 0° and 40°. Fig. 2A shows that the E_m values of the cytochrome C555 titrations do not vary significantly with pH. Table II shows the values of ΔG°, ΔH°, and ΔS° for the cytochrome C555 half-cell reaction.

As the redox potential is lowered through the region from +100 mV to −50 mV, the secondary electron donor switches from cytochrome C555 to cytochrome C552[3,4,13,15]. Fig. 5A presents a typical redox titration of this changeover. Fig. 2B demonstrates that the E_m value of +11 mV for this titration does not vary

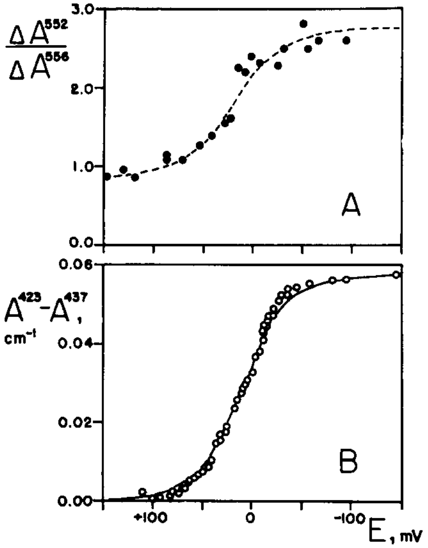

Fig. 5. A. Changeover from cytochrome C555 photooxidation to cytochrome C552 photooxidation as a function of the redox potential. The ordinate indicates the ratio of the flash-induced absorbance change at 552 nm to that at 556 nm. (See ref. 15 for spectra of the two cytochromes.) Monochromator band pass, 2.0 nm. Bacteriochlorophyll, 58 μM. Redox buffer B; pH 8.1. The dashed curve represents a theoretical one-electron titration curve with $E_{m8.1} = +20$ mV. B. Redox titration of cytochrome C552 under steady-state conditions. No actinic illumination. Bacteriochlorophyll, 30 μM. PMS, 100 μM; methylene blue, 100 μM; pH 7.7. Solid curve represents a theoretical one-electron titration with $E_{m7.7} = +9$ mV. The figure shows a slight distortion of the titration from the Nernst equation. This distortion was not observed consistently.

significantly with pH. As Fig. 5B indicates, the changeover from cytochrome C555 photooxidation to cytochrome C552 photooxidation correlates with the chemical reduction of a low-potential cytochrome, with $E_{m7.7} = +9$ mV.

The primary electron acceptor, X

At low redox potentials, the amount of cytochrome oxidation which occurs on a single flash serves as an indication of the redox state of X. The closed circles in Fig. 6 depict a representative titration over the potential region from +50 mV to −200 mV, and Parts B and C of Fig. 2 show the midpoint potentials of similar titrations at a variety of pH values. Gramicidin D (0.6 μg/ml) had no effect on the titration of X.

A second indicator of the reduction of X is an increase in the yield of BChl fluorescence. Fig. 2C also shows E_m values which were obtained from titrations of BChl fluorescence. E_m values obtained by the two methods agreed well. The best estimates of $E_{m7.7}$ at 22° are −134 mV, obtained from titrations of first-flash cytochrome oxidation, and −131 mV, obtained from titrations of BChl fluorescence yield. The E_m decreases with increasing pH by 36 mV per pH unit (Fig. 2).

Fig. 3C gives the temperature dependence of the $E_{m7.7}$ values of the X titrations. The $E_{m7.7}$ decreases linearly with increasing temperature between 0° and 40°. At 22°, the best estimate of $E_{m7.7}$ from these data is −116 mV. Table II gives values of ΔG°, ΔH°, and ΔS° for the half-cell reduction of X. Our values of $E_{m7.7}$ at 22° compare with those reported by CUSANOVICH *et al.*[3] ($E_{m7.5} = -130$ mV), DUTTON[4] ($E_{m7.8} = -135$ mV), SEIBERT *et al.*[5] ($E_{m7.4} = -129$ mV), and CRAMER[6] ($E_{m8.0} = -160$ mV).

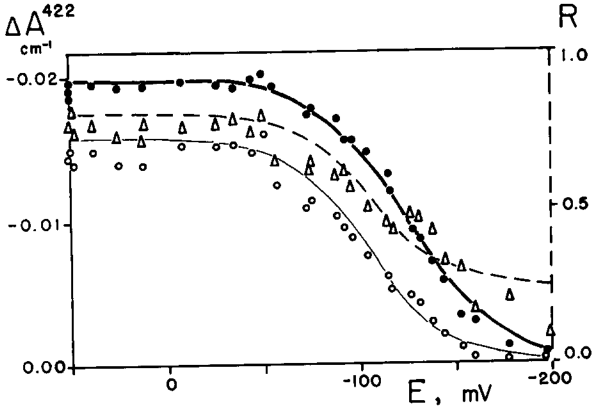

Fig. 6. Redox titrations of X and Y. Measurements of cytochrome photooxidation at 422 nm following one and two saturating laser flashes. Bacteriochlorophyll, 47 μM. Redox buffer A. pH 7.70. ●, absorbance changes following the first flash. The bold solid curve is a theoretical one-electron titration curve with $E_{m7.7} = -125$ mV. ○, absorbance changes following a second flash, one msec after the first. The fine solid curve is a theoretical curve for z (see DISCUSSION) in which the pool size of Y is assumed to be 1.7 times that of X, and the $E_{m7.7} = -125$ mV for X and −95 mV for Y. △, the ratio, R, of the cytochrome photooxidation which occurs on the second flash to that which occurs on the first. The dashed curve is a theoretical $z/(1-x)$ curve for the same assumptions regarding X and Y as above.

The secondary electron acceptor, Y

An indicator of the redox state of Y is the cytochrome oxidation which occurs on a second flash 1 msec after the first flash. The open circles in Fig. 6 show a titration of cytochrome oxidation on the second flash. Variations in the time interval between the first and second flashes from 300 μsec to 10 msec yielded midpoint potentials which were indistinguishable (less than ±10 mV) from the one in Fig. 6. Fig. 2B presents midpoint potentials which were observed at a variety of pH values, and Fig. 3C shows the pH 7.7 midpoint potentials at several temperatures. The midpoint potential decreases with increasing pH by 38 mV per pH unit, and decreases linearly with increasing temperature between 0 and 40°. Again, gramicidin D (0.6 μg/ml) had no effect on these titrations.

The difference between the midpoint potential for the second flash titration and the true E_m of Y depends on the pool size of Y. A more detailed treatment of this relationship is presented in the DISCUSSION. A satisfactory account of the data of Fig. 6 is made if one assumes a pool of 1.5 to 2.0 molecules of Y per molecule of X, and if the reduction of Y is a single-electron reaction. If this pool size is correct, the true E_m of Y is about 10 mV more positive than the midpoint potential of the titration. The best estimates of the actual $E_{m7.7}$ of Y at 22° are −101 mV, obtained from the data of Fig. 2B, and −83 mV, obtained from the data of Fig. 3C. In the calculations of the ΔG° and ΔH° values for the reduction of Y (Table II), the same +10 mV correction has been applied to all of the observed $E_{m7,7}$ values from Figs. 2B and 3C.

Additonal information on the size of the Y pool came from measurements of P870 photooxidation after three flashes. The experiment of Fig. 7 considers the rate of X^- oxidation in the presence of a partially-reduced pool of Y. The figure shows the amount of P870 oxidation which occurs on a third flash, as a function of the delay between the second and third flashes. As measured by the effect of the third flash, X^- reoxidation is half-maximal when the interval is about 45 μsec.; it is complete by about 1 msec.

In this experiment, the ambient redox potential is low enough so that cyto-

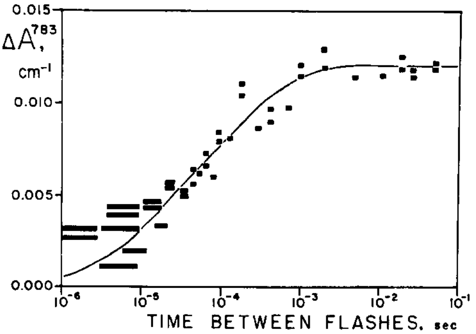

Fig. 7. P870 oxidation following a third flash as a function of the time interval between the second and third flashes. The time interval between the first and second flashes was 10 msec. Ferric EDTA, 50 μM. Bacteriochlorophyll, 46 μM. pH 7.70. The redox potential ranged between +220 and +270 mV. Due to the 5-μsec width of the third flash, the time interval between the second and third flashes becomes vague at time intervals below 40 μsec. The vagueness is expressed in the figure as the bars at short time. Ordinarily, the time interval was taken to be that from the second flash to the peak of the third flash. The abscissa scale is logarithmic.

chrome C555 is almost exclusively in the reduced form initially. Little P870 remains oxidized after the first and second flashes, because P870$^+$ reduction by cytochrome C555 is rapid[1]. After the third flash, however, substantial P870$^+$ remains. P870$^+$ reduction is slow after the third flash, because the first two flashes have oxidized essentially all of the cytochrome C555 (ref. 16). The amount of P870 photooxidation which results from the third flash is approximately 65 percent of the total P870 present (compare Figs. 1 and 7). In order for the primary photochemical reaction to work three times, X$^-$ must transfer an electron to the Y pool twice. Therefore, at least two equivalents of Y must be available.

The effect of PMS on the titration of Y

The presence of 100 μM PMS has no effect on the redox titrations of X, cytochrome C555 ,or cytochrome C552. However, if one replaces redox Buffer A (which contains ferric EDTA, but no PMS) with Buffer B (which contains PMS, but not ferric EDTA), a marked change occurs in the low-potential titration of cytochrome oxidation on a second flash. The apparent midpoint potential shifts from -101 to $+37$ mV at pH 7.7, and the pH dependence of the midpoint potentials changes from -38 to -56 mV per pH unit (Figs. 2B and 2C). Titrations in which ferric EDTA was omitted from buffer A behaved like those in which ferric EDTA was present, indicating that these changes involve a specific effect of PMS. The influence of PMS is evident in a titration which we described previously[15].

The basis of the effect of PMS on the titration of Y is not clear. Redox titrations of PMS itself, shown in Fig. 2C for several pH values, indicate that the chemical reduction of free PMS does not correlate directly with the loss of cytochrome oxidation on the second flash. Although PMS appears to replace Y as a secondary electron acceptor in subchromatophore preparations from *Chromatium*[13], there is no indication that this occurs in chromatophores. Double-flash experiments demonstrated that the addition of 100 μM PMS to chromatophore suspensions had no effect on the rate of oxidation of X$^-$ at redox potentials ranging from $+200$ to $+250$ mV.

DISCUSSION

The cytochrome C555 titrations

Previous experiments[16] have indicated that the cytochrome C555 heme which undergoes oxidation after a single flash at high redox potentials is indistinguishable from that which responds to a second flash. Fig. 4A, however, shows that cytochrome oxidation on the first flash persists at higher redox potentials than does cytochrome oxidation on the second flash. Rather than ascribe the different titration curves to chemical differences between the two cytochromes, we propose the following model.

Assume that each photosynthetic unit contains one P870 complex and two indistinguishable cytochrome C555 hemes, and that cytochrome C555 oxidation will occur on the first flash if either one or both of the two hemes in a given photosynthetic unit is reduced prior to the first flash. If c is the fraction of the cytochrome C555 which is in the reduced form, then the probability that at least one heme in a given photosynthetic unit is present in the reduced state is $1-(1-c)^2$, or

$2c - c^2$. At redox potentials high enough that essentially no reduced cytochrome C552 is present, cytochrome oxidation on the second flash will occur in a given photosynthetic unit only if both cytochrome C555 hemes are reduced prior to the first flash. The probability that a second flash will elicit cytochrome oxidation is then c^2.

If oxidation altered the net charge on cytochrome C555, one would expect titration of one of the two hemes to perturb the E_m of the other. On the other hand, if the electrostatic interaction between the two cytochrome C555 hemes does not change appreciably upon oxidation of the cytochrome, c would obey the Nernst equation. In that case, the potential at which $2c - c^2$ is half-maximal is 44 mV above that at which c^2 is half-maximal. The true E_m of the cytochrome, at which $c = 0.5$, should lie half-way between the midpoint potentials of the $2c - c^2$ and c^2 curves. The cytochrome C555 titrations agree well with this prediction (Figs. 4A and 4B, Table II).

The cytochrome C552 titrations

Bartsch[21] and Bartsch and Kamen[22] have isolated from *Chromatium* a soluble cytochrome ("soluble C552") with the spectral properties of cytochrome C552 and $E_{m7.0} = +10$ mV. Figs. 5A and 5B and the mean $E_{m7.7}$ values in Table II support the following conclusions. (1) Soluble C552 is identical with the low-potential cytochrome in chromatophores. (2) The E_m of this cytochrome is the same whether the cytochrome is soluble or bound. (3) If reduced cytochrome C552 is available, P870$^+$ oxidizes cytochrome C552 in preference to cytochrome C555. Comparison of Figs. 4B and 5B suggests that, if cytochromes C555 and C552 have comparable extinction coefficients, both cytochromes are present in the chromatophores in pools of approximately the same size. This observation differs from that of Thornber[23], who found 5 to 7 heme equivalents of cytochrome C552 and 2 of cytochrome C555 per reaction center in a subchromatophore preparation from *Chromatium*. The reasons for this difference are not yet clear.

Whether an analysis similar to the one described above for cytochrome C555 photooxidation applies to the redox titrations of cytochrome C552 is not clear. The unknown factors which result in the preferential oxidation of cytochrome C552 by P870$^+$ would complicate such an analysis.

The X and Y titrations

At redox potentials below +250 mV, each photosynthetic unit contains at least one reduced cytochrome available for both the first and second flashes. If the two flashes are more than a few μsec apart, the cytochrome photooxidation which occurs on each flash is a measure of the amount of X which is in the oxidized state prior to that flash[17]. If a fraction, x, of X is reduced at a given redox potential, the cytochrome oxidation on the first flash will be proportional to $(1 - x)$.

Suppose that, at the same potential, the fraction of Y in the reduced state prior to the first flash is y, and that electron transfer between X$^-$ and Y has reached equilibrium. Then, if this transfer is a one-electron reaction,

$$K = \frac{(1-x)(Ny)}{(x)(N-Ny)} = \frac{(1-x)(y)}{(x)(1-y)} \tag{1}$$

where K is the equilibrium constant, and N is the pool size of Y relative to that of X. During the first flash, X becomes completely reduced[17], and the electron transfer reaction between X^- and Y seeks a new equilibrium. Because the amount of cytochrome photooxidation which occurs on the second flash reaches a plateau within 1 msec (Fig. 6 of ref. 17), we assume that equilibrium is attained by this time, and that further reactions of Y^- require a much longer time. If the fraction of X^- which becomes reoxidized is z, the new equilibrium is given by,

$$K = \frac{(z)\,(Ny + z)}{(1 - z)\,(N - Ny - z)} \qquad (2)$$

Thus, z in Eq. 2 determines the amount of cytochrome photooxidation which results from a second flash 1–10 msec after the first (Fig. 6), and z is a function of N as well as of the redox potential.

On the assumption that x and y are governed by the Nernst equation, and the further assumption of trial values of N and K, one can use Eq. 2 to calculate titration curves for z and the ratio $z/(1 - x)$. A satisfactory fit to the cytochrome photooxidation data was obtained for N values of 1.5 to 2.0, and $K = 3.7$ (Fig. 6). From the value of K, the $E_{m7.7}$ of Y is approximately 33 mV more positive than that of X. The same assumptions also allowed an adequate fit of the data which were obtained at other temperatures and pH values. N values greater than 2.0 gave a much poorer fit, for any assumptions on K. If $N = 2.0$, the E_m of Y is about 10 mV more positive than the midpoint of the z titration.

Although our results are consistent with a value of 2.0 for N, one should note that N need not be integral. The reaction center population might well be heterogeneous, with some centers containing one equivalent of Y and others, two. Alternatively, several reactions centers might share a common pool of Y molecules which is somewhat larger than the number of reaction centers.

If the electron transfer reaction between X^- and Y involves two electrons, so that the stoichiometry is $2X^- + Y \rightleftarrows 2X + Y^{2-}$, the following equation replaces Eq. 2:

$$K' = \frac{(z)^2\,(Ny + \frac{1}{2}z)}{(1 - z)^2\,(N - Ny - \frac{1}{2}z)} \qquad (2')$$

This equation did not allow a satisfactory fit to the data of Fig. 6 for any value of N, regardless of the assumptions concerning K'.

The rate of the transfer of an electron from X^- to Y after a second flash (Fig. 7) does not differ significantly from the rate which prevails after the first flash (Fig. 6 of ref. 17). This agreement is surprising, in that the concentration of oxidized Y must differ by a factor of approximately two in the two experiments, if the pool size of Y is twice that of X. Apparently the rate of X^- reoxidation is independent of the concentration of oxidized Y.

The cytochrome C555 oxidation kinetics are similar, in that the rate of oxidation of the second cytochrome C555 heme is the same as that for the first heme[16]. Evidently, the rate of cytochrome oxidation does not depend on the concentration of reduced cytochrome C555.

pH dependence of the titrations

The pH dependence of the electron transfer reactions is of considerable interest because of the possibility that light-induced pH gradients play a critical role in photophosphorylation[24-27]. The redox titrations of P870$^+$, X, and Y all have a similar dependence on pH; an uptake of approximately $\frac{1}{2}$H$^+$ per electron occurs in the half-cell reduction of each of the three species (Fig. 2 and Table II). However, no net uptake or release of protons would occur on the transfer of an electron from X$^-$ to Y, and little or none on the primary reaction between P870 and X.

Because the E_m values of cytochrome C555 and C552 are independent of pH (Fig. 2 and Table II), an uptake of approximately $\frac{1}{2}$H$^+$ per electron must accompany the oxidation of either cytochrome by P870$^+$. CHANCE *et al.*[28] have, in fact, detected H$^+$ uptake following exposure of *Chromatium* chromatophores to a single short flash. However, the half-time of the H$^+$ uptake which they observed was much longer than the half-times of the cytochrome oxidations.

Thermodynamic properties of the photochemical reactions

If C and P represent the reduced forms of cytochrome C555 and P870, and C$^+$ and P$^+$ their oxidized forms, one can symbolize a photosynthetic unit as (C P X Y). Then, disregarding protons, the cytochrome C555 redox titrations of Fig. 4 follow the half-cell reaction,

$$(\mathrm{C^+PXY}) + e^- \rightleftharpoons (\mathrm{CPXY}) \qquad (3)$$

Similarly, the redox titrations of X in Fig. 6 follow the half-cell reaction.

$$(\mathrm{CPXY}) + e^- \rightleftharpoons (\mathrm{CPX^-Y}) \qquad (4)$$

Algebraic combination of these two reactions gives the following dismutation:

$$2(\mathrm{CPXY}) \rightleftharpoons (\mathrm{C^+PXY}) + (\mathrm{CPX^-Y}) \qquad (5)$$

The oxidized cytochrome and reduced X which are created in Reaction 5 occupy separate photosynthetic units. The photochemical reaction of interest, however, is not Reaction 5, but

$$(\mathrm{CPXY}) \rightleftharpoons (\mathrm{C^+PX^-Y}) \qquad (6)$$

This is a disproportionation reaction which generates the oxidized and reduced carriers within the same photosynthetic unit.

Presumably, X$^-$ and C$^+$ are much closer together in the products of Reaction 6 than in those of Reaction 5. If, as the symbolism suggests, X$^-$ has one more negative charge than X, and C$^+$ has one more positive charge than does C, electrostatic interactions could stabilize the products of Reaction 6 relative to those of Reaction 5. This means that the redox titrations would provide only an upper limit to the amount of free energy which is stored in the photochemical reaction.

Consider, instead of Reaction 3, the half-cell reaction

$$(\mathrm{C^+PX^-Y}) + e^- \leftrightharpoons (\mathrm{CPX^-Y}) \qquad (7)$$

Algebraic combination of Reactions 4 and 7 yields the desired disproportionation Reaction 6. Thus, a comparison of the cytochrome C555 E_m values from Titrations 3 and 7 should give the free energy of charge stabilization in (C$^+$ P X$^-$ Y).

If the assumptions in the preceding paragraph are valid, one might expect the E_m for Titration 7 to be below the E_m of Titration 3. In fact, we found no significant influence of the redox state of X on the E_m of cytochrome C555.

SCHEJTER AND MARGALIT[29] have reported recently that the net charge on horse heart cytochrome *c* decreases upon oxidation of the ferrocytochrome in the presence of NaCl. They attribute the charge decrease to the selective binding of Cl^- to the ferricytochrome. If changes in ion binding occur on the oxidation of cytochrome C555 or on the reduction of X, the transfer of an electron from the cytochrome to X might leave unchanged the net electrostatic interaction between the two electron carriers. This reasoning would also explain the observation (Fig. 4B) that the steady-state titrations of the two cytochrome hemes fit the Nernst equation.

The preceding considerations appear to justify the view that algebraic combinations of the redox titration data allow calculations of the free energy changes in the photochemical reactions. Fig. 8 summarizes the results of these calculations, giving standard state values of $\Delta G°$, $\Delta H°$, and $-T\Delta S°$ for the primary and secondary photochemical processes. The most surprising feature of the results is that an entropy decrease of about 36 cal·°K^{-1}·mole^{-1} accounts for all of the free energy which is stored in the transfer of an electron from cytochrome C555 or P870 to X or Y. The molecular basis of the entropy decrease and its significance for the mechanism of photophosphorylation remain for exploration.

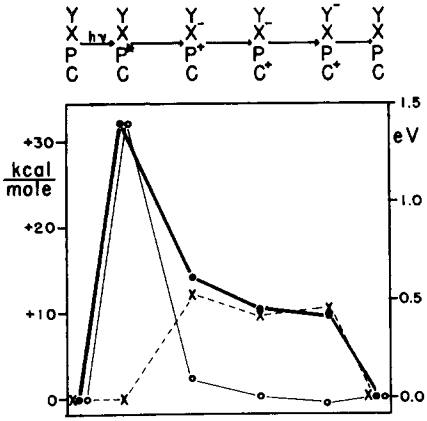

Fig. 8. Cumulative changes in free energy, enthalpy, and entropy in the photochemical reactions in *Chromatium*. Values callulated from data of Table II. ●, $\Delta G°$; ○, $\Delta H°$; ×, $-T\Delta S°$. The reactions are given at the top of the figure, using symbols which are defined in the DISCUSSION. The entropy change for the initial excitation of P870, (C P X Y) $\xrightarrow{h\nu}$ (C P* X Y), is assumed to be zero.

One should keep in mind that the redox titrations reflect quasi-equilibrium states, and might not reveal transient high-enthalpy states of the electron carriers. Any transmembrane electrical potential, for example, would relax during the periods when one adjusts the redox potential. The insensitivity of the titrations to gramicidin D may illustrate this point. By facilitating the flow of monovalent cations across the chromatophore membrane, gramicidin would accelerate the collapse of a transmembrane electrical potential[30]. However, experiments with a sensitive and rapid flash calorimeter appear to confirm our conclusion that the energy of a flash of light is degraded immediately to heat, and that any free energy storage must take the form of an entropy decrease[31].

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