Conversion of Light into Chemical Free Energy through Chlorophyllin-Sensitized Photoreduction of Oxidized Nicotinamide–Adenine Dinucleotide Phosphate by Cytochrome *c**

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ABSTRACT: A model system is presented in which red light is utilized to drive the enzymatic photoreduction of oxidized nicotinamide-adenine dinucleotide phosphate by ferrocytochrome *c* in the presence of aggregated chlorophyllin a. The only net chemical change in this system is the oxidation of 2 moles of ferrocytochrome *c* for each mole of oxidized nicotinamide-adenine dinucleotide phosphate reduced. The path of electron transfer in this model reaction is: ferrocytochrome *c* → aggregated chlorophyllin a → oxidized nicotinamide–adenine dinucleotide phosphate reductase → oxidized nicotinamide–adenine dinucleotide phosphate. The amount of light converted into chemical free energy in this model reaction is 8.4 kcal/mole of electrons transferred from ferrocytochrome *c* to oxidized nicotinamide–adenine dinucleotide phosphate, corresponding to a photoelectromotive force of 0.36 V. The average quantum efficiency is found to be 5.3%.

The pioneering work of Krasnovsky (1948) stimulated extensive experimental work on chlorophyll- or chlorophyllin-sensitized model photoredox systems (Brin and Krasnovsky, 1959; Vernon, 1961; Vernon *et al.*, 1965; Kassner and Kamen, 1967; Brody *et al.*, 1968; Eisenstein and Wang, 1969). But of these model systems only a few lead to a net conversion of light into chemical free energy, the rest are actually light-activated oxidation–reduction reactions which are thermodynamically favorable in the dark. For example, chlorophyll-sensitized oxidation of ferrocytochrome *c* by oxygen (Brin and Krasnovsky, 1959) or ferrohemochrome (Brody *et al.*, 1968) is thermodynamically favorable in the the dark. Although the chlorophyllin-sensitized photoreduction of $NADP^+$ by ascorbate in the presence of $NADP^+$-reductase (Vernon *et al.*, 1965) may have involved a net conversion of light into chemical free energy, the ascorbate causes a partial degradation of the sensitizer, as shown by the permanent decrease of the Soret peak, and hence the experimental data cannot be used to demonstrate unambiguously the conversion of light into chemical free energy.

The present model system consists of an aqueous solution of ferri- and ferrocytochrome *c*, $NADP^+$, chloroplast $NADP^+$-reductase, and aggregated chlorophyllin a in phosphate buffer at pH 7. Upon illumination by red light under strictly anaerobic conditions, the reduction of $NADP^+$ by an equivalent amount of ferrocytochrome *c* took place as the only net chemical change in the system. Under the present experimental contions, electrons are driven by light from ferrocytochrome *c* to $NADP^+$ *via* aggregated chlorophyllin a and $NADP^+$-reductase against an electrochemical potential of 0.36 V.

Experimental Section

Materials. Chloroplast $NADP^+$-reductase was prepared from fresh spinach (Huang *et al.*, 1969) by the procedure of Forti and Sturani (1968). The $NADP^+$-reductase so obtained showed distinct absorption maxima at 275, 385, and 456 mμ as well as two hidden maxima at 308 and 485 mμ. The ratio of absorbances at 456 and 275 mμ was found to be $A_{456}/A_{275} = 0.11$. The activity of the $NADP^+$-reductase was checked by the catalytic photoreduction of $NADP^+$, ferricyanide, and 2,6-dichlorophenolindophenol (Sigma Chemical Co.), respectively, with spinach chloroplasts prepared by the procedure of Allen and coworkers (1958).

Ethyl chlorophyllide a was prepared from fresh spinach by L. Fabry in our laboratory, using the procedure of Holt and Jacobs (1954). The phytyl group of natural chlorophylls was first replaced by the theyl group, using the chlorophyllase of *Ailanthus altissima* as a catalyst. The ethyl chlorophyllides a and b were then separated chromatographically on a sucrose column and crystallized twice. The purified ethyl chlorophyllide a was subsequently converted into chlorophyllin a by means of KOH and crystallized from anhydrous methanol according to the procedure of Oster and coworkers (1964). All operations were carried out in the dark. In methanol solution, the chlorophyllin a showed absorption maxima at 416 and 655 mμ; in aqueous phosphate buffer at pH 7.0, at 416 and 642 mμ.

Horse heart cytochrome *c* (Grade IV), $NADP^+$ (monosodium salt, Sigma Grade), and NADPH (tetrasodium salt, Sigma Grade) were purchased from Sigma Chemical Co. All the other chemicals are of the Reagent Grade.

Assay of Cytochrome c. The ferricytochrome *c* was reduced to the ferrous form by hydrogen gas in the presence of palladium black. Since the ferricytochrome *c* showed a broad absorption band at 535 mμ, whereas the ferrocytochrome *c* showed two sharp absorption peaks at 520 and 550 mμ, respectively, the concentrations of the oxidized and reduced forms of cytochrome *c* could be conveniently determined spectrophotometrically. The millimolar extinction coefficients, ε, of cyto-

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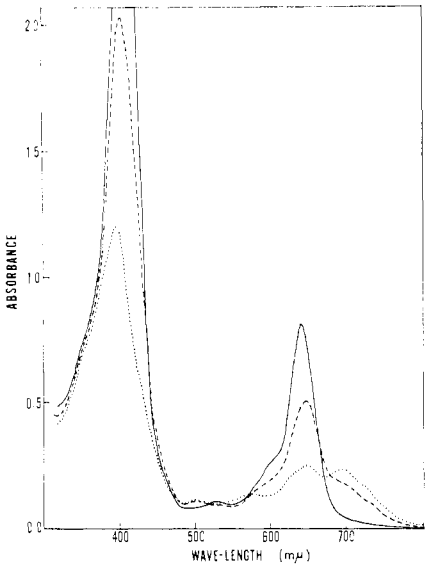


FIGURE 1: Absorption spectra of chlorophyllin a at different stages of light-activated aggregation in 0.1 M aqueous phosphate buffer at pH 7.0.(——) Fresh solution before illumination, (----) after illumination by red light for 30 min, and (· · · ·) after illumination by red light for 10 hr.

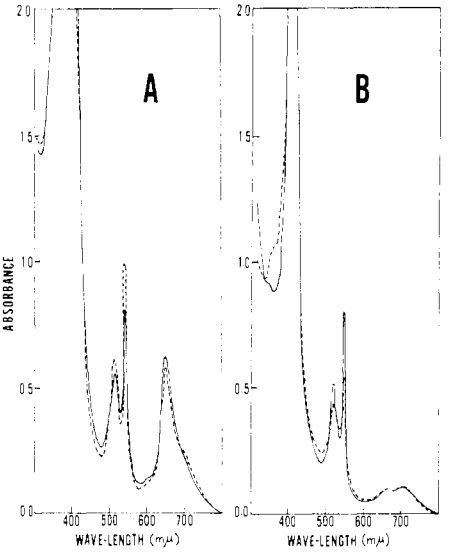


FIGURE 2: Photoredox reactions between chlorophyllin a and cytochrome *c* in 0.1 M aqueous phosphate buffer at pH 7.0. (A) Spectra of a solution containing ferricytochrome *c*, ferrocytochrome *c*, and freshly prepared monomeric chlorophyllin a: ——, before illumination; ----, after illumination by red light for 20 min. (*B*) Spectra of a solution containing ferricytochrome *c*, ferrocytochrome *c*, and an equilibrium mixture of aggregated chlorophyllin a: ——, before illumination; ----, after illumination by red light for 15 min.

chrome *c* at the above wavelengths are listed (Margoliash and Frohwirt, 1959) as: for ferricytochrome *c*, ϵ_{520} 10.2, ϵ_{535} 10.9, and ϵ_{550} 9.0; and for ferrocytochrome *c*, ϵ_{520} 15.9, ϵ_{535} 7.2, and ϵ_{550} 27.7.

If the ratio of absorbances A_{550} and A_{520} of a given sample of cytochrome *c* at 550 and 520 mμ, respectively, is m, the concentration c_t of total cytochrome *c* and the fraction X in the ferrous form are given by

$$c_t = \frac{A_{550}}{9.0(1 - X) + 27.7(X)} \quad \text{and}$$

$$X = \frac{10.2m - 9.0}{(27.7 - 9.0) - m(15.9 - 10.2)} = \frac{10.2m - 9.0}{18.7 - 5.7m}$$

Assay of NADPH. Since the broad absorption band of NADPH at 340 mμ (ϵ_{340} 6.0) overlaps with the strong Soret bands of cytochrome *c* and chlorophyllin a, the concentration of NADPH was assayed by measuring its fluorescence at 460 mμ by means of either a Perkin-Elmer Hitachi Model MPF-2A fluorescence spectrophotometer or a Farrand Model A-2 photoelectric fluoremeter. Using 340-mμ actinic light, the fluorescence intensity at 460 mμ was found to be proportional to the concentration of NADPH in solution within ±0.5%.

Because of the high absorbance of cytochrome *c* in the reaction mixture at 460 mμ, a correction to the observed NADPH fluorescence for this "inner filter" is necessary. This was made as follows. Let us assume that at the end of a photoredox experiment the observed fluorescence intensity at 460 mμ due to NADPH in the mixture was Y_1, and that by adding a known amount of pure NADPH to this mixture the fluorescence intensity was increased to Y_2. If the same amount of NADPH added to a colorless buffer solution causes a fluorescence increment equal to Y_3, the corrected value for Y_1 is $Y_{1\,cor} = Y_1(Y_3/(Y_2 - Y_1))$.

Measurement of the Photoredox Reaction. The sensitized photoredox reactions were carried out under anaerobic conditions (air pressure from 10^{-4} to 10^{-3} mm) in an optical cell fused to an all-glass manifold. A 150-W tungsten lamp was used for the actinic light source. An 1.5-in. thick cold-water filter and a Corning C. S. No. 25 red filter were placed between the tungsten lamp and the reaction vessel at room temperature.

Measurement of Quantum Yield. The quantum yield of the photoredox reaction was determined by the method of Warburg and Schoken (1962), using the methyl chlorophyllide sensitized photooxidation of thiourea by O_2 at 25.0 ± 0.1° as the standard. The volume of O_2 consumed by the actinometric solution was determined by the displacement of a micrometer plunger, which was attached to the closed arm of the Warburg apparatus, required to restore the pressure to its initial value.

Results

Aggregation of Chlorophyllin a. Chlorophylls are notorious for their tendency to aggregate (Krasnovsky and Kosobutskaya, 1953; Thomas, 1962). Recently Gulyayev and Litvin (1967) suggested from their spectrometric studies that at least eight different aggregated forms of chlorophyll a exist in nature.

The detailed structure of chlorophyllin a has not yet been conclusively established. Oster and coworkers (1964) showed from their infrared data that the molecule has several free carboxylate groups but no cyclopentanone ring. Following these investigators, we also found that light accelerates the

TABLE I: Chlorophyllin$_n$-Sensitized Photoreduction of NADP$^+$ by Cytochrome c.[c]

Expt	Initial [NADP$^+$] (μM)	[Total Cyt c] (μM)	Initial [Ferricyt c] (μM)	[Reductase] (μM)	Illumination by Red Light, Sensitizer[a] (min)	Final [Ferricyt c] (μM)	Final [NADPH][b] (μM)
1	117	34.4	5.0	0.24	30, (chl)$_n$	15.7	5.0
2	97	25.9	4.3	0.26	30, (chl)$_n$		3.8
3	97	0.0	0.0	0.26	30, (chl)$_n$		0.7
4	97	47.7	0.0	0.26	40, no (chl)$_n$	0.0	0.0
5	97	47.7	0.0	0.26	40 in the dark, no (chl)$_n$	0.0	0.0
6	97	37.0	24.0	0.00	15, no (chl)$_n$	24.0	0.0
7	97	37.0	24.0	0.00	15 in the dark, no (chl)$_n$	24.0	0.0
8	125	27.5	9.3	0.00	30, (chl)$_n$		0.09

[a] When the sensitizer (chl)$_n$ was used, its concentration was approximately 5×10^{-6} mole of monomer equiv/l. [b] No NADPH was present at the beginning of any experiment. [c] All experiments were performed in 0.1 M aqueous phosphate buffer at pH 7.0 and 23°.

aggregation of chlorophyllin a with an accompanying change in the absorption spectrum of the system in 0.1 M aqueous phosphate buffer at pH 7.0. Under strictly anaerobic conditions when the photooxidation of chlorophyllin a by air was eliminated, the only effect of illuminating the system with red light (wavelength > 600 mμ) was to increase the rate of approaching the aggregation equilibrium. The absorption spectra of chlorophyllin a in its aqueous solution at different stages of the light-accelerated aggregation in a typical experiment are shown in Figure 1. After the solution was illuminated by red light anaerobically for 10 hr, no further change in its absorption spectrum could be observed. At this stage, the addition of a small excess of solid KOH to the system immediately changed the absorption spectrum back to the pattern of unaggregated chlorophyllin a at high pH. This last observation demonstrates the reversibility of the light-activated aggregation of chlorophyllin a.

Photoredox Properties of Chlorophyllin a. When a solution of freshly prepared, essentially monomeric form of chlorophyllin a and cytochrome c in aqueous 0.1 M phosphate buffer at pH 7.0 was illuminated by red light anaerobically, the cytochrome was further reduced. But when the same experiment was repeated with the aggregated form of chlorophyllin a, the cytochrome was further oxidized. The results of two typical experiments are shown in Figure 2.

Chlorophyllin$_n$-Sensitized Enzymatic Photoreduction of NADP$^+$ by Cytochrome c. Since the photoredox reactions involving the monomeric form of chlorophyllin a cannot be conveniently separated from the light-activated aggregation reaction, an equilibrium mixture of the aggregated forms of chlorophyllin a was used as the sensitizer in all of the following experiments. For convenience, let us represent this mixture of aggregated forms of chlorophyllin a by (chl)$_n$.

When an aqueous solution of NADP$^+$, (chl)$_n$, chloroplast NADP$^+$-reductase, ferri-, and ferrocytochrome c in phosphate buffer at pH 7 was illuminated by red light under anaerobic conditions, a net electron transfer from cytochrome c to NADP$^+$ took place. In the absence of either the NADP$^+$-reductase or (chl)$_n$, very little or no NADPH was formed. The results of two typical experiments (expt 1 and 2) and six control experiments (expt 3–8) are summarized in Table I.

TABLE II: Stoichiometry of the Model Photoredox Reaction.

Expt	[Cyt c Oxidized] (μM)	[NADPH Formed] (μM)	Stoichiometric Ratio
1	8.76	4.80	1.8
2	10.2	4.77	2.1
3	6.95	3.26	2.1
4	12.8	5.80	2.2
5	9.09	4.22	2.2
6	7.00	3.80	1.8
7	8.29	3.50	2.4
8	6.70	3.52	1.9
9	10.7	5.00	2.1
10	5.35	2.67	2.0
		Av	2.06

TABLE III: Quantum Yield Measurements.

Expt	μmoles of Photons Absorbed by the Model System	μmoles of Ferrocyt c Oxidized by NADP$^+$	Quantum Yield (%)
1	0.338	0.0150	4.4
2	0.295	0.0150	5.8
3	0.295	0.0150	5.8
4	0.253	0.0135	5.3
		Av	5.3

TABLE IV: Conversion of Light into Chemical Free Energy by the Model Photoredox Reaction.

Expt	Final Concentrations of Reactants				$\Delta G/n$ (kcal/Faraday)
	[$NADP^+$] (μM)	[NADPH] (μM)	[Ferricyt *c*] (μM)	[Ferrocyt *c*] (μM)	
1	116	4.80	8.76	14.0	8.24
2	120	4.77	10.2	57.7	8.83
3	110	3.26	6.95	30.3	7.51
4	96.0	5.80	12.8	4.63	9.10
5	99.8	4.22	9.09	4.67	8.91
6	89.7	3.80	7.00	4.76	8.75
7	93.5	3.50	8.29	3.65	8.81
8	93.5	3.52	6.70	17.3	7.65
9	112	5.00	10.7	15.3	8.37
10	83.3	2.67	5.35	12.5	8.00

In expt 3, a small amount of NADPH was formed in the absence of cytochrome *c*. The reductant in this case was presumably the $(chl)_n$ itself, since its absorption spectrum after the illumination was appreciably different from the initial spectrum. In all the other experiments with $(chl)_n$ listed in Table I, there was no detectable difference in the absorption spectra of the $(chl)_n$ before and after the illumination.

The stoichiometric relationship between the cytochrome *c* oxidized and $NADP^+$ reduced was established by the ten experiments listed in Table II. The average result

$$\frac{[\text{cyt } c \text{ oxidized}]}{[\text{NADPH formed}]} = 2.1 \pm 0.18$$

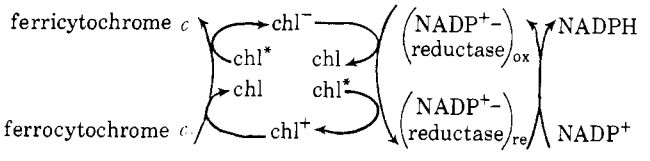
is consistent with the over-all net reaction

$$2\text{ferricytochrome } c + NADP^+ + H^+ \xrightarrow[NADP^+\text{-reductase}]{h\nu,(chl)_n} 2\text{ferrocytochrome } c + NADPH$$

Quantum Yield. The results of quantum yield measurements are summarized in Table III. A Corning C. S. No. 25 filter, which transmits practically all visible light of wavelength above 600 mμ, was used to filter the actinic light from the tungsten lamp.

Discussion

The sum of the above experimental results shows that the path of electron transfer in the present model photoredox reaction can be represented by the following scheme, in which either chl^- or chl^+ or both participate in the electron transfer where chl represents chlorophyllin a in its ground electronic state, chl* chlorophyllin a in its excited electronic state, chl^+ the oxidized chlorophyllin radical, chl^- the reduced chlorophyllin radical, and the subscripts ox and re designate the oxidized and reduced states, respectively. Presumably Förster's type of energy transfer (Förster, 1949) can take place between different monomer units chl and chl* of the same chlorophyllin aggregate as well as between different chlorophyllin species in the solution. This reaction scheme is also consistent with the extensive electron spin resonance data obtained by Tollin and Green (1962, 1963), by Tollin and coworkers (1965), by Banerjee and Tollin (1966), by Chibisov and coworkers (1966), by Fuhrkop and Mauzerall (1968), by McElroy and coworkers (1969), as well as the circular dichroism data on the reaction centers of photosynthetic bacteria (Sauer *et al.*, 1968) and electron spin resonance data on chloroplast $NADP^+$-reductase (Huang *et al.*, 1969).



Since the midpoint reduction potentials of ferricytochrome *c* and $NADP^+$ in aqueous solutions at pH 7 are 0.255 and -0.32 V, respectively, the amount of light converted into chemical free energy per mole of electrons transferred from ferrocytochrome *c* to $NADP^+$ under the conditions at the end of each experiment is equal to

$$\Delta G/n = (E_{NADP^+} + E_{cyt\ c})F$$
$$= -\left\{\left(-0.32F - \frac{RT}{2}\ln\frac{[NADPH]}{[NADP^+]}\right) - \left(0.26F - RT\ln\frac{[\text{ferrocyt } c]}{[\text{ferricyt } c]}\right)\right\}$$
$$= 0.58F + \frac{RT}{2}\ln\left\{\frac{[NADPH][\text{ferricyt } c]^2}{[NADP^+][\text{ferrocyt } c]^2}\right\},$$

where *F* is the Faraday constant, *R* the gas constant, and [NADPH], [$NADP^+$], [ferricyt *c*], and [ferrocyt *c*] the molar concentrations of the corresponding reacting species at the end of the experiment. The values of $\Delta G/n$ calculated from the above experiments are summarized in Table IV. The average value, $\Delta G/n = 8.4$ kcal/faraday, corresponds to a photoelectromotive force of 0.36 V.

In view of the fact that not all the molecular species of chlorophyllin a in the equilibrium mixture represented by

$(chl)_n$ are equally effective as sensitizers and that the over-all photoredox reaction discussed above involves three successive electron transfer steps, the observed average quantum yield of 0.053 is remarkably high indeed.

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