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Action Spectrum and Quantum Requirements for the Photoreduction of Cytochrome *c* with Spinach Chloroplasts*

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ABSTRACT: The photoreduction of cytochrome *c* in the presence of intact chloroplasts occurs with a high quantum efficiency, using reduced trimethyl-*p*-benzoquinone ($TMQH_2$) as reductant and in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This reaction has a requirement of 2 quanta absorbed per electron transferred to cytochrome *c* for exciting light in the wavelength region from 620 to 680 mμ; the quantum requirement then falls to 1 quantum per electron at wavelengths greater than 700 mμ. These results confirm the conclusion of Vernon and Shaw [Vernon, L. P., and Shaw, E. R. (1965), *Biochemistry 4*, 132] that this oxidation–reduction reaction is mediated by chloroplast pigment system I in the presence of DCMU. The quantum requirement of unity observed at long wavelengths shows that the reaction probably occurs with the maximum efficiency obtainable. The evaluation of the action spectrum for cytochrome *c* reduction together with that for the chloroplast Hill reaction [Sauer, K., and Park, R. B. (1965), *Biochemistry 4*, 2791], which is probably photocatalyzed by pigment system II, strongly suggests that there is no appreciable transfer of electronic excitation energy between the two pigment systems in spinach chloroplasts.

The two light reactions apparently interact only at the chemical level of photosynthetic electron transport. A model is presented which rationalizes this conclusion by the physical separation of the two pigment systems on opposite sides of the chloroplast lamellar unit.

The recent studies of Vernon and Shaw (1965) demonstrated that the photoreduction of cytochrome *c* by whole chloroplasts is stimulated by the addition of various hydroquinones, including reduced trimethyl-*p*-benzoquinone ($TMQH_2$).[1] The stimulation is only partially decreased in the presence of DCMU, a potent inhibitor of oxygen evolution by chloroplasts. This finding suggested to Vernon and Shaw that these hydroquinones serve as electron donors for the long wavelength pigment system I of chloroplasts.

The present investigation seeks to determine by means of its action spectrum whether this photoreduction is a system I reaction. The evidence strongly suggests that such is the case, and the data are used to derive the spectral absorption of pigment system I. The action spectrum for cytochrome *c* reduction by chloroplasts is found to be similar to that reported previously for the photoreduction of NADP with $DCPIPH_2$ and ascorbate in the presence of DCMU, a known system I reaction (Hoch and Martin, 1963; Sauer and Biggins, 1965). Furthermore, the consideration of the action spectrum for cytochrome *c* reduction together with that for the chloroplast Hill reaction (Sauer and Park, 1965) provides strong evidence to support the hypothesis that there is no transfer of electronic excitation energy between the two pigment systems in spinach chloroplasts.

Materials and Methods

Chloroplasts were prepared either from fully grown commercial spinach leaves or from 6–8 week-old plants grown from seed in a growth chamber, as described

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[1] Abbreviations used in this work: $TMQH_2$, reduced trimethyl-*p*-benzoquinone; NADP ($NADPH_2$), nicotinamide-adenine dinucleotide phosphate; DCPIP ($DCPIPH_2$), 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

previously (Sauer and Park, 1965). Horse heart cytochrome *c*, obtained from Sigma Chemical Co., St. Louis, was dried under vacuum over $Mg(ClO_4)_2$. Samples of the dried cytochrome were oxidized with ferricyanide and reduced with dithionite, and a $\Delta\epsilon_{549.5m\mu}^{red\text{-}ox}$ of 1.9×10^4 l. mole^{-1} cm^{-1} was observed. This is in good agreement with values of 1.9–2.1 $\times$ 10^4 l. mole^{-1} cm^{-1} in the literature (Paléus and Neilands, 1950; Massey, 1959). DCMU was obtained from Du Pont, Wilmington.

$TMQH_2$ was prepared by reduction of TMQ (K & K Laboratories, Jamaica, N. Y.) with dithionite in a two-phase reaction mixture of water and toluene. Further purification was achieved by crystallization of the $TMQH_2$ from toluene or diethyl ether followed by sublimation *in vacuo*. A partial reoxidation to TMQ was observed in air either upon recrystallization or upon solution of the solid $TMQH_2$ in ethanol to prepare the reagent solution. Thus, the reaction mixture contained some TMQ at the start of the photoreaction.

Reaction rates were obtained by continuously monitoring the absorbance of the reaction mixture at 549.5 mμ (the α-band maximum for cytochrome *c*) while the sample was being irradiated from the side with longer wavelength light. A Cary Model 14 spectrophotometer with a modified Model 1462 scattered-transmission accessory was used, as described by Sauer and Biggins (1965). Exciting light was obtained from a Bausch and Lomb monochromator with supplementary cut-off filters; and light intensity measurements, corrected for reflection losses, were made using a calibrated photovoltaic cell.

The reaction mixture contained potassium phosphate, pH 7.5, 0.05 M; sucrose, 0.1 M; and the following in μmoles/liter: cytochrome *c*, 50; $TMQH_2$, 85; and DCMU, 0.9. (The $TMQH_2$ and DCMU were made up in ethanol solutions, which were diluted 100- and 200-fold, respectively, in the reaction mixture.) A sufficient amount of the chloroplast preparation was added in the dark at the start of each measurement to give an absorbance of chlorophyll at 678 mμ of 0.3–0.7 for a 1-cm path. It was found that a solution containing cytochrome *c* and $TMQH_2$ becomes deactivated slowly upon standing in the dark; consequently, a fresh reaction mixture (2 ml) was prepared for each wavelength of exciting light studied. Altogether, seven different chloroplast preparations were used in the study. These generally exhibited no loss in activity for periods up to 5 hr in the dark at 0°. All measurements were made in air, except where noted, and at room temperature. In no case was the photoreaction carried to more than 15% conversion of the cytochrome *c*.

Results

The photoreduction was studied as a function of light intensity over a 5- to 30-fold range at each of 24 wavelengths in the region from 620 to 740 mμ. At each wavelength, the calculated quantum requirements were found to increase somewhat with increasing incident light intensity. As in previous studies (Sauer and Biggins, 1965; Sauer and Park, 1965), the measured quantum requirements were extrapolated linearly to zero light intensity. The zero intensity quantum requirements are summarized as a function of wavelength in Figure 1 for the two chloroplast preparations studied most extensively. Three other preparations gave results in excellent agreement with these; in the other two there was a partial inactivation of the chloroplasts during the isolation procedure and quantum requirements about twice as large were obtained, but with the same wavelength dependence.

The system usually exhibits a fairly strong back reaction in the dark following illumination, although for two preparations of chloroplasts it was virtually absent. The rate of the back reaction, when it occurred, was proportional to the percentage conversion of the cytochrome *c*, and all photochemical rates reported are corrected for the appropriate interpolated dark reaction. An attempt to reduce this back reaction by purging the reaction mixture with nitrogen proved unsuccessful; no change in rates of either the back reaction or the photoreduction was observed.

A reaction mixture in which the chloroplasts had been heated to 65° for 3 min, conditions known to destroy system I activity (Vernon and Zaugg, 1960; Rumberg and Witt, 1964), exhibited no cytochrome *c* photoreduction when it was illuminated at 680 mμ. This is taken as an indication that the photoreduction requires the integrity of the chloroplast structure and not just the presence of the pigments.

Methylamine is known to uncouple the chloroplast Hill reactions from photophosphorylation and to lower the quantum requirements for the Hill reaction at moderate light intensities (Sauer and Park, 1965). In the case of cytochrome *c* reduction, however, methylamine (10 μmoles ml^{-1}) had no effect on the rates of either the photoreduction or the back reaction.

Discussion

On the basis of their observation that the photoreduction of cytochrome *c* by $TMQH_2$ in the presence of chloroplasts is largely DCMU-insensitive, Vernon and Shaw (1965) proposed that in the presence of DCMU the reaction is catalyzed by pigment system I. The action spectrum presented in Figure 1 of this paper strongly supports their conclusion. The action spectrum has a fairly constant zero-intensity quantum requirement of 2 quanta absorbed/equivalent of cytochrome *c* reduced for wavelengths from 620 to 680 mμ. At longer wavelengths there is a decrease in quantum requirement to 1.0 quantum/equivalent at about 710 mμ, which remains constant to 740 mμ. The very high efficiency (low quantum requirement) at wavelengths longer than 700 mμ is a characteristic feature of system I catalyzed reactions by higher plant chloroplasts. It differs strongly from the action spectrum of the Hill reaction using DCPIP or ferricyanide, where the quantum requirement is 2–3 from 640 to 680 mμ and then increases as much as 10-fold at wavelengths longer

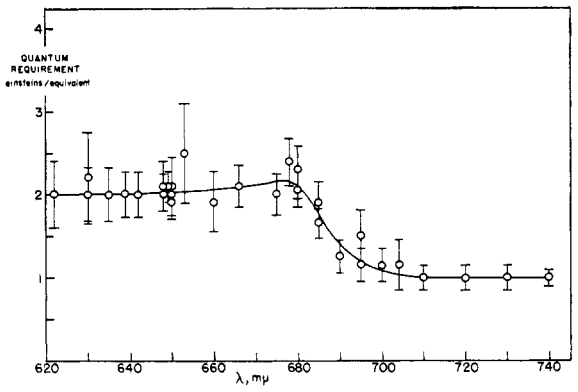

FIGURE 1: Action spectrum for the reduction of cytochrome *c* by $TMQH_2$ using spinach chloroplasts (two different preparations). The quantum requirements are values obtained from extrapolations to zero light intensity at each wavelength.

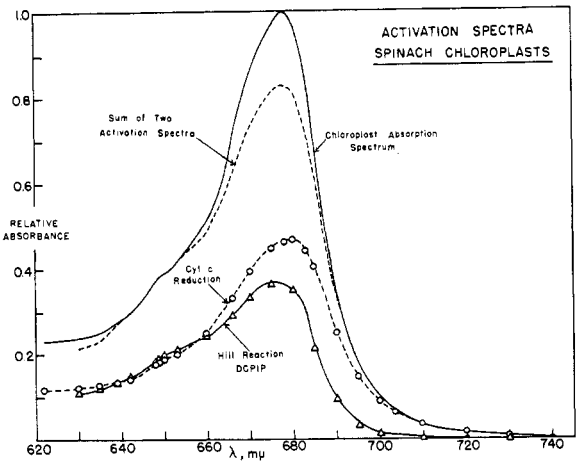

FIGURE 2: Absorption spectra of pigments responsible for cytochrome *c* reduction by $TMQH_2$ in presence of DCMU (○, and lower dashed curve) and for the DCPIP Hill reaction (△, and lower solid curve; data from Sauer and Park, 1965) by spinach chloroplasts. Upper solid curve gives the normalized absorption spectrum of spinach chloroplasts, corrected for light scattering (Sauer and Biggins, 1965). Upper dashed curve is the sum at each wavelength of the two lower curves.

than 690 mμ (Sauer and Park, 1965). The action spectrum for cytochrome *c* reduction by chloroplasts is similar to that for the chloroplast-catalyzed photoreduction of NADP by ascorbate coupled with a small amount of $DCPIPH_2$ and in the presence of DCMU (Hoch and Martin, 1963; Sauer and Biggins, 1965). The ascorbate–$DCPIPH_2$ couple provides electrons in lieu of water and does not, apparently, require the participation of pigment system II.

The quantum requirements for the cytochrome *c*–$TMQH_2$ reaction are uniformly lower than those observed previously for NADP reduction using ascorbate–$DCPIPH_2$, and we feel that the former are more representative of the optimum photochemical potentiality of pigment system I. The higher quantum requirements for NADP reduction by ascorbate–$DCPIPH_2$ probably result from the presence of a cyclic as well as a noncyclic pathway for this reaction. Cyclic photophosphorylation is mediated both by ferredoxin, which is an essential cofactor for the NADP photoreduction by chloroplasts (Tagawa *et al.*, 1963; Arnon *et al.*, 1964), and by $DCPIPH_2$–ascorbate (Gromet-Elhanen and Avron, 1963; Shen *et al.*, 1963).

Quantum yields extrapolated to zero light intensity for the cytochrome *c* reduction and for the DCPIP Hill reaction (Sauer and Park, 1965) are given in Table I for the various wavelengths of exciting light. Figure 2 shows activation spectra obtained by the technique of Sauer and Park (1965) of multiplying the observed zero-intensity quantum yields (reciprocal of the quantum requirement) at each wavelength by the normalized total absorbance of spinach chloroplasts. The activation spectra obtained in this way represent the absorption spectra of the "active pigments," *i.e.*, that portion of the total pigments responsible for the sensitization of the particular photoreaction being studied. The maximum of the cytochrome *c*–$TMQH_2$ activation spectrum (system I) occurs at about 680 mμ and is virtually identical with the normalized absorption spectrum (topmost curve in Figure 2) at longer wavelengths. The maximum is at 675 mμ for the DCPIP Hill reaction and is nearly zero at wavelengths longer than 700 mμ. This reaction has been assigned to system II by Rumberg *et al.* (1962) on the basis of absorption change measurements. Even if system I participates as well in this reaction, its activation spectrum is probably very close to the absorption spectrum of system II; *e.g.*, see Sauer and Park (1965). The difference between the two activation spectra in Figure 2 in the region of maximal absorption by chlorophyll *b* (*ca.* 650 mμ) is slight. It would be hazardous to assign chlorophyll *b* primarily to pigment system II on the basis of this evidence.

In the last column of Table I are given values for the sum of the quantum yields for the two reactions at each wavelength. A curve representing the sum of the activation spectra is also presented in Figure 2. This synthesized spectrum is very similar, both in shape and magnitude, to the observed absorption spectrum, giving strong confirmation to the proposal of Sauer and Park (1965) that the activation spectra are really the absorption spectra of the respective pigment systems and that the sum of the activation spectra is simply the over-all measured absorption spectrum. This would not necessarily be the case if there were appreciable transfer of electronic excitation energy from one pigment system to the other. If electronic energy transfer were possible, the sum of quantum yields could be as high as 2 at some wavelengths, particularly in the wavelength region from 620 to 685 mμ where both systems appear to absorb com-

TABLE 1: Quantum Yields for Cytochrome *c* Reduction by $TMQH_2$ and for the DCPIP Hill Reaction of Spinach Chloroplasts at Various Wavelengths.

Wavelength (mμ)	Quantum Yields (equivalents/einstein absorbed)		
	ϕ_{Cyt}	ϕ_{DCPIP}[a]	$\phi_{Cyt} + \phi_{DCPIP}$
622	0.50		
630	0.50		
635	0.50	0.44	0.94
639	0.50	0.49	0.99
642	0.50	0.50	1.00
648	0.50	0.51	1.01
650	0.49	0.52	1.01
653	0.49	0.51	1.00
660	0.48	0.48	0.96
666	0.48	0.42	0.90
670	0.47	0.39	0.86
675	0.46	0.38	0.84
678	0.46	0.37	0.83
680	0.48	0.36	0.84
683	0.53		
685	0.58	0.30	0.88
690	0.72	0.26	0.98
695	0.83	0.18	1.01
700	0.88	0.13	1.01
704	0.94		
710	1.00	0.08	1.08
720	1.00	0.08	1.08
730	1.00	0.04	1.04
740	1.00		

[a] Data taken from the results of Sauer and Park (1965).

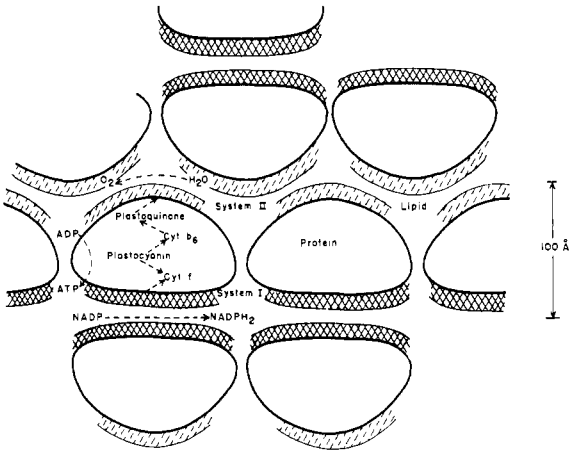

FIGURE 3: A model of a cross section of the chloroplast lamellar system, showing a proposed physical separation of the two pigment systems and of the products of their photoreactions. Portions of several identical quantasome units are sketched.

parably. If no electronic energy transfer is possible, then the sum cannot be greater than 1.0 *at any wavelength.* The data in the last column of Table I are quite clear on this point. The values observed are all 1.0 ± 0.1 in the wavelength region from 620 to 740 mμ, with the exception of the region around the absorption maximum at 678 mμ, where the sum falls to 0.83. (We now have evidence that the low values in this region result from the monochromator band width of 10 mμ used routinely in these and the previous studies. In this particular region of the spectrum, the sample is appreciably more transparent to light in the wings of the band of wavelengths incident on it than to those near the center. When measurements are made for various band widths of 680 mμ exciting light and extrapolated to zero band width, the quantum yield sum increases from 0.84 to 0 97.) We have every reason to believe that the cytochrome *c*-$TMQH_2$ reaction is operating at optimum efficiency, since only one absorbed quantum is required for each electron transferred at wavelengths longer than 700 mμ. It is not easy to postulate a simple mechanism whereby this intrinsic quantum yield is then reduced to 0.5 at shorter wavelengths, at the same time permitting efficient electronic energy transfer to occur. The authors believe that the simplest explanation lies in the postulate that electronic energy transfer can occur only within each pigment system and not between them and that the coupling of the two pigment systems occurs only at the chemical level.

This postulate is not one which is necessarily obvious *a priori.* It is known that electronic excitation can be transferred over fairly large distances (30–40 A) between molecules of chlorophyll *a* in solution (Watson and Livingstone, 1950; Weber, 1960). Such processes should occur *in vivo* as well. On the basis of evidence from fluorescence studies, Robinson (1964) has suggested that electronic energy transfer from system II to system I is inefficient at room temperature but becomes more important at low temperatures where the photochemical reactions are blocked. If the absorption and emission oscillators of the pigment molecules of the two pigment systems *in vivo* are oriented unfavorably with respect to one another, then radiative transfer would have a low probability. This seems unlikely in view of the absence of any strong orientation of the bulk of the pigment molecules in chloroplasts or active lamellar fragments isolated from them, using the tests of fluorescence polarization (Arnold and Meek, 1956; Goedheer, 1957) or dichroism (Goedheer, 1955, 1957; Olson *et al.*, 1962; Sauer and Calvin, 1962; Sauer, 1965). A much simpler explanation is that the two pigment systems are physically separated *in vivo* by a distance greater than 30–40 A, and that the medium separating them is one which does not especially facilitate the transfer of electronic excitation energy in competition with chemical processes at room temperature.

A model for pigment ordering within the chloroplast, consistent both with the requirement of separated pig-

ment systems and with the current picture of chloroplast lamellar structure, especially as elucidated by the electron microscope studies of Park and co-workers (Park and Pon, 1961, 1963; Park and Biggins, 1964; Park, 1965), is shown in Figure 3. The model consists of a lamellar array of the order 100 A thick made up of a planar assembly of quantasome particles, with the molecules of the two pigment systems imbedded on opposite faces of the planar array and separated by a matrix containing protein and colorless lipid. Separations of at least 30–40 A would be perfectly feasible in such a model. The intervening lipoprotein matrix would contain many of the intermediate cofactors (cytochromes, quinones, plastocyanin, phosphorylation sites, etc.) which couple the two pigment systems at the chemical level. If adjacent lamellae are in an antiparallel arrangement, shown in the model and supported by the electron microscopic studies, then the model has the additional advantage suggested by Robinson (1964) of providing for the physical separation of the powerful reductants (chloroplast ferredoxin, $NADPH_2$) normally produced by pigment system I reactions and the powerful oxidants (molecular oxygen, etc.) which are products of pigment system II reactions.

Other models are possible. For example, if the respective pigment systems were always separately located on quantasomes in different regions of the lamellar array, which is consistent with the views of Olson *et al.* (1961) and of Gross *et al.* (1964), the requisite separation in space would be accomplished. We know of no compelling evidence, either morphological or photochemical, in support of this hypothesis. On the other hand, it makes the problem of chemical communication between the two pigment systems that much more difficult.

The antiparallel lamellar hypothesis illustrated in Figure 3, where the pigment systems are on opposite sides or the same quantasomes and the central region provides the principal pathway of chemical communication between them, appeals to us as being simple conceptually and compatible with the notion of the chloroplast as an array of photosynthetic units roughly the size of quantasomes.

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