*Biochimica et Biophysica Acta*, 387 (1975) 115–128


BBA 46896

# THE KINETICS AND SPECIFICITY OF ELECTRON TRANSFER FROM CYTOCHROMES AND COPPER PROTEINS TO P700

P. MUIR WOOD and D. S. BENDALL

*Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW (U.K.)*

(Received August 16th, 1974)

## SUMMARY

The rates of electron transfer to P700 from plastocyanin and cytochrome *f* have been compared with those from three other *c*-type cytochromes and azurin, a copper protein resembling plastocyanin. Three different disruptive techniques were used to expose P700; digitonin, Triton X-100 and sonication. The following rate constants were measured at 25 °C, pH 7.0, with digitonin-treated chloroplasts: plastocyanin, $8 \cdot 10^7\ M^{-1} \cdot s^{-1}$; red-algal cytochrome *c*-553, $1.9 \cdot 10^7\ M^{-1} \cdot s^{-1}$; *Pseudomonas* cytochrome *c*-551, $8 \cdot 10^6\ M^{-1} \cdot s^{-1}$; azurin, $\lessapprox 3 \cdot 10^5\ M^{-1} \cdot s^{-1}$; cytochrome *f*, $\lessapprox 2 \cdot 10^4\ M^{-1} \cdot s^{-1}$; mammalian cytochrome *c*, $\lessapprox 2 \cdot 10^4\ M^{-1} \cdot s^{-1}$. For electron transfer from plastocyanin, the effects of ionic strength, pH and temperature were also studied, and saturation effects found in earlier work were avoided by a full consideration of the various secondary reactions and inclusion of superoxide dismutase. The relative rates are discussed in relation to photosynthetic electron transport.

## INTRODUCTION

There is good evidence that in chloroplasts cytochrome *f* and plastocyanin are involved in electron transfer between plastoquinone and P700, the reaction-centre pigment of photosystem I, but some uncertainty remains concerning the precise pathway of electron flow. One of us has recently measured the rate coefficient for electron transfer between purified cytochrome *f* and plastocyanin [1]. This was compared with the rates of electron transfer between plastocyanin and three other *c*-type cytochromes, and the reactions were also studied with plastocyanin replaced by azurin, a similar copper protein. None of these rate constants exceeded that for reaction between cytochrome *f* and plastocyanin.

We have now extended this approach by comparing the rates of photo-oxidation of these four cytochromes and two copper proteins by disrupted chloroplast

Abbreviations: cytochrome $c_{mam}$, horse heart cytochrome *c*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonate; MES, 2-(*N*-morpholino)-ethanesulphonate; MeV, methyl viologen; PC, plastocyanin.

systems, with which we assume that direct electron transfer is possible between the soluble protein and P700. There is good evidence that P700 is located on the inside of the thylakoid membranes [2], and therefore interaction between P700 and exogenous redox proteins is only possible after the membranes have been broken. Several disruptive techniques can be used, with care, to expose P700 to exogenous proteins, for example sonication [3] or treatment with detergents such as Triton X-100 [4] or digitonin [5]. We have based our experiments on the plastocyanin assay system of Plesničar and Bendall [6], and in agreement with them have found digitonin to be the most satisfactory disruptive agent. Treatment with Triton as described below gives behaviour similar to that with digitonin, but absolute rates of about a factor of two lower, and whereas digitonin gives rates that are independent of detergent concentration over a wide range, higher Triton levels apparently cause further disruption leading to low rates with plastocyanin and a loss of specificity [7, 8]. Sonication gives lower rates than digitonin [9], probably because only part of the P700 is left exposed; the ability of some sonicated preparations to phosphorylate [2] indicates the presence of closed vesicles. We have found that supposedly identical preparations of fractionated photosystem I particles have widely varying activities, and therefore freshly prepared chloroplasts were used throughout without any attempt at fractionation after detergent had been added.

Although reactions between soluble proteins and P700 have been the subject of frequent study, the results have mainly been only semi-quantitative, and the possibility of cyclic electron flow involving the superoxide anion ($O_2^{\cdot-}$) has never been considered. We have been able to avoid saturation effects encountered in earlier work [5, 10–13] and have found high rates and clear specificity. The implications are discussed with particular reference to the roles of plastocyanin and soluble algal cytochrome in photosynthetic electron transport.

## METHODS

Chloroplasts were prepared from peas (variety Feltham First) by a method similar to that of Walker [14]. The peas were grown for about two weeks in moist vermiculite. 40 g of leaves were blended for 5 s with a "Polytron" (Kinematica GmbH, Luzern) with 200 ml of medium containing 0.33 M mannitol, 25 mM $K_2HPO_4$, 25 mM $NaH_2PO_4$, 10 mM NaCl and 2 mM $MgCl_2$ at 0° C. After filtration through muslin the homogenate was centrifuged for 2 min at $4000 \times g$. The chloroplasts were resuspended in the grinding medium and centrifuged for 30 s at $500 \times g$ to remove aggregates and cell wall debris. Chlorophyll was assayed by the method of Arnon [15].

The P700 content of chloroplast preparations was determined at 25 °C from the difference spectrum of ascorbate (reduced) minus ferricyanide (oxidised), assuming a difference in molar extinction coefficient $\Delta\varepsilon(\text{red}-\text{ox}) = 6.4 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for the peak near 700 nm, and an isosbestic point at 727 nm [16]. The difference spectra were recorded with a sensitive split-beam spectrophotometer (made by the Johnson Research Foundation, University of Pennsylvania) with chloroplasts at about 25 $\mu$g chlorophyll/ml, usually pretreated with digitonin as described below. The chlorophyll: P700 ratio varied from 430 to 600 in different preparations.

Cytochromes, plastocyanin and azurin were purified and estimated as described by Wood [1]. The sources were as follows: cytochrome *f* and plastocyanin from pars-

ley (*Petroselinum sativum*), azurin and cytochrome *c*-551 from *Pseudomonas fluorescens* and red-algal cytochrome *c*-553 from *Plocamium coccineum*. Horse heart cytochrome *c* (cytochrome $c_{mam}$) was purchased from BDH Chemicals Ltd. For most experiments the proteins were required in the reduced form; cytochromes *f* and *c*-553 were in the reduced state as prepared and the others were reduced by addition of a 10-fold excess of ascorbate, followed by dialysis for plastocyanin and azurin, and passage of the solution through a Sephadex G-25 column in the case of the cytochromes.

Superoxide dismutase was prepared from bovine erythrocytes by the procedure of McCord and Fridovich [17], except that the chromatography was omitted. After dialysis against 2.5 mM potassium phosphate (pH 7.4) the dismutase was freeze dried and stored at −20 °C. It was assayed by the cytochrome *c* method of McCord and Fridovich [17] with the following modifications: a 50 mM sodium borate buffer at pH 8.5 was used [18], including 50 mM KCl and $10^{-4}$ M EDTA, and 1 μl of catalase was added to avoid complications due to $H_2O_2$ [19]. Aliquots of superoxide dismutase were added to inhibit cytochrome *c* reduction and $k(O_2^- + \text{superoxide dismutase})/k(O_2^- + \text{cytochrome } c) = 1.6 \cdot 10^4$ was assumed [18, 20].

Catalase was purchased from the Boehringer Corp. as a crystalline suspension with stated activity $7.8 \cdot 10^5$ units/ml.

The photochemical reactions were monitored with a dual wavelength recording spectrophotometer as described by Wood [1]. Actinic light was provided by a 500 W tungsten lamp projector (Aldis) arranged to give uniform illumination of one side of the cuvette, and since low chlorophyll concentrations were used ($\lesssim 1$ μM) the illumination was reasonably uniform across the cuvette. The actinic beam was filtered through an infrared heat filter and a red glass filter (Schott RG 630). The photomultiplier was protected by complementary filters: a 1 cm pathlength of saturated $CuSO_4$ and a green Kodak Wratten No. 40 gelatin filter. The wavelengths used were 510 and 565 nm for plastocyanin and azurin reactions (absorption by the filters prevented use of higher wavelengths), and values of about 536 and 553 nm for the cytochrome reactions, which were selected so as to maximise the absorption change upon oxidation.

Except where otherwise stated measurements were made at 25 °C in a 10 mM phosphate buffer, pH 7.0, with 80 mM NaCl added to give an ionic strength of 0.1. In most runs digitonin (Analar, from BDH Chemicals Ltd) was dissolved in the buffer at 2 mg/ml by heating to 90 °C, followed by filtration through a sintered glass funnel; the solution was used within 2–3 h. The reagents were mixed in a water-jacketed 1 $cm^2$ spectrophotometer cuvette to give a total volume of about 2.5 ml. In a typical experiment chloroplasts were added to the buffered digitonin to give a final concentration of not more than 1 μg chlorophyll/ml, and followed by about 2 μM reduced cytochrome or cuproprotein, 100 μM methyl viologen, 2 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (from a 1 mM solution in ethanol, and added to inhibit photosystem II), 50 nM superoxide dismutase and 2 μl catalase. The rationale for these concentrations of cofactors is explained below; higher values were used in experiments with high concentrations of plastocyanin. After illumination for about 30–60 s it was frequently convenient to establish a base line by adding excess potassium ferricyanide to give complete oxidation. The temperature was recorded at the end of a run, and heating caused by the actinic light was not more than a few tenths of a degree.

Two other disruptive techniques were used in some experiments. For treatment with the neutral detergent Triton X-100 the procedure was to dilute the chloroplasts (at 0 °C) to 50 μg chlorophyll/ml and add sufficient 5 % Triton solution to give a Triton : chlorophyll molar ratio of 20 : 1, corresponding to about 1 mM Triton. For disruption by sonication 5 ml of chloroplasts, diluted to 50 μg chlorophyll/ml with the usual buffer, was sonicated for 1 min with a Dawe "Soniprobe" type 1130A fitted with a 1.2 cm diameter probe. During sonication the temperature of the sample was kept close to 0 °C by cooling with an alcohol/solid $CO_2$ bath. After treatment with Triton or sonication an appropriate volume of the disrupted chloroplasts was added to the buffer in the cuvette, as described above except for the absence of digitonin.

## RESULTS

### *Requirements for simple kinetics*

In the case of photo-oxidation of plastocyanin by photosystem I the sequence of reactions under aerobic conditions and with methyl viologen present is believed to be as follows:

$$PC_{red} + P700_{ox} \xrightarrow{k_1} PC_{ox} + P700_{red} \quad (1)$$

$$P700_{red} + P430_{ox} + h\nu \xrightarrow{k_2} P700_{ox} + P430_{red} \quad (2)$$

$$P430_{red} + MeV + H^+ \xrightarrow{k_3} P430_{ox} + MeVH \quad (3)$$

$$MeVH\cdot + O_2 \xrightarrow{k_4} MeV + O_2^- + H^+ \quad (4)$$

$$2H^+ + 2O_2^- \xrightarrow{k_5} H_2O_2 + O_2. \quad (5)$$

For our studies it was essential that (1) should be rate limiting, and that all cyclic reactions should be avoided. The most likely cyclic reactions are:

$$P430_{red} + PC_{ox} \xrightarrow{k_6} P430_{ox} + PC_{red} \quad (6)$$

$$MeVH\cdot + PC_{ox} \xrightarrow{k_7} MeV + H^+ + PC_{red} \quad (7)$$

$$O_2^- + PC_{ox} \xrightarrow{k_8} O_2 + PC_{red}. \quad (8)$$

These reactions will now be considered in turn. In (2), P430 denotes the postulated primary acceptor of photosystem I [21]. After absorption of a quantum of light the charge transfer takes place in less than 20 ns [22] but under the steady illumination used here the effective rate of reoxidation of reduced P700 will be a function of the light intensity and can be expressed in $s^{-1}$. For reaction (3), Hiyama and Ke [23] found $k_3 = 9.6 \cdot 10^6\ M^{-1} \cdot s^{-1}$. For reaction (4) Farrington et al. [24] report $k_4 = 7.7 \cdot 10^8\ M^{-1} \cdot s^{-1}$, one of the fastest reactions known for radical + $O_2$ (this provides a reason for preferring methyl viologen to alternative photosystem I acceptors), and in view of the rate of (4) and the high $[O_2]$/[plastocyanin] ratio used throughout, reaction (7) can be ignored. (Similar reasoning shows that direct reduction of $P700_{ox}$ by $P430_{red}$ or MeVH · can also be ignored.) Reaction (5) can occur spontaneously, in which case it is second order in $[O_2^-]$ (ref. 25) and therefore slow at low concentrations of superoxide. It can also be mediated by superoxide dismutase which gives $k = 2.3 \cdot 10^9\ M^{-1} \cdot s^{-1}$, first order in both $[O_2^-]$ and [dismutase]

(ref. 20). Chloroplasts contain superoxide dismutase at about 1 molecule per 3000 molecules chlorophyll [26] but this was inadequate for the present experiments.

It was necessary to measure the rates of (6) and (8) in order to establish what ratios of [methyl viologen]/[plastocyanin] and [superoxide dismutase]/[plastocyanin] would eliminate cyclic reactions. To measure $k_6$, we studied the rate of photoreduction of plastocyanin by osmotically shocked chloroplasts (no detergent) in the presence of superoxide dismutase and catalase. The reduction was inhibited by methyl viologen, providing evidence that the reaction is mediated by photosystem I, as expected from current ideas about sites of interaction of added electron acceptors [27]. Fig. 1 shows a pseudo-first-order plot for photoreduction of plastocyanin, and from the slope $k_6 = 2 \cdot 10^6\ M^{-1} \cdot s^{-1}$ could be deduced. Similar rates of reduction were found for cytochromes $c_{mam}$ and $c$-551 in the presence of superoxide dismutase [28] or under anaerobic conditions. The photoreduction of plastocyanin by shocked chloroplasts was discovered by Katoh [29] before the oxidation by P700 was known; he also found that plastocyanin stimulated the rate of photoreduction of added cytochrome $c_{mam}$.

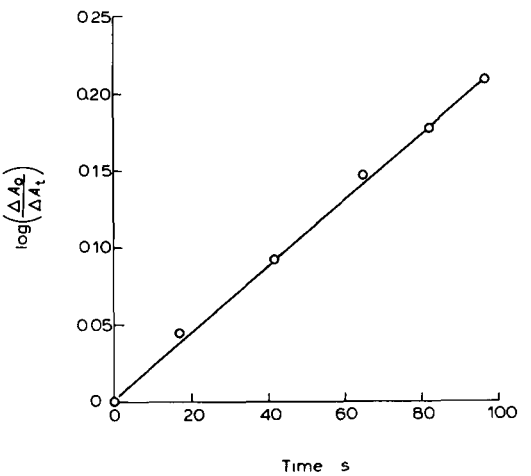


Fig. 1. First-order plot for photoreduction of plastocyanin by osmotically shocked chloroplasts. The buffer was pH 7.0 phosphate, ionic strength 0.1, and the total volume was 2.43 ml. The reaction mixture contained 4.0 $\mu$M plastocyanin (oxidised), 50 nM superoxide dismutase, 2 $\mu$l catalase and chloroplasts at 1.04 $\mu$g chlorophyll/ml, giving 2.2 nM P700.

For measurement of the rate of reaction (8), the procedure used to assay superoxide dismutase was repeated with plastocyanin instead of cytochrome $c_{mam}$. This was done in the pH 8.5 medium used for assaying superoxide dismutase and also in the phosphate buffer (pH 7.0) used for chloroplast experiments. With the measurements of Klug et al. [20] for $O_2^-$ + superoxide dismutase ($k = 1.9 \cdot 10^9\ M^{-1} \cdot s^{-1}$ at pH 8.5, $2.3 \cdot 10^9\ M^{-1} \cdot s^{-1}$ at pH 7.0), our data led to $k_8 = 1.0 \cdot 10^5\ M^{-1} \cdot s^{-1}$ at pH 8.5, and $2.6 \cdot 10^5\ M^{-1} \cdot s^{-1}$ at pH 7.0. These values are very similar to the rates for $O_2^-$ + cytochrome $c_{mam}$: $k = 1.1 \cdot 10^5\ M^{-1} \cdot s^{-1}$ at pH 8.5 [18] and $3 \cdot 10^5\ M^{-1} \cdot s^{-1}$ at pH 7.0 (deduced from refs 18, 20, 30).

On the basis of these measurements, superoxide dismutase was generally added to give [plastocyanin]/[superoxide dismutase] $\lesssim$ 100, and methyl viologen was added in a generous excess over plastocyanin. A small amount of catalase was also added, since while $H_2O_2$ is unreactive towards plastocyanin and similar proteins, its decomposition can be efficiently catalysed by transition metal impurities, and may then give rise to reactive oxidation states [31].

It was assumed that this rationale, developed for plastocyanin and also valid for cytochrome $c_{mam}$ (which has $k_6$ negligible and $k_8$ comparable with plastocyanin), would also hold for the other proteins used. The obvious sign of trouble would be a slowing down of the pseudo-first-order rate as significant amounts of oxidised protein accumulated, and this was not found in any case.

*Photo-oxidation of plastocyanin*

Fig. 2 shows a typical pseudo-first-order plot for plastocyanin photo-oxidation by digitonin treated chloroplasts. The slope of such a plot equals $k_1 \cdot [P700]\ s^{-1}$ from which $k_1$ can be derived. The mean value for the rate constant in the presence of digitonin was $8 \cdot 10^7\ M^{-1} \cdot s^{-1}$ at 25 °C, pH 7.0 and ionic strength 0.1, with variation by $\pm 20\ \%$ in different chloroplast preparations.

Experiments with up to 30 $\mu$M reduced plastocyanin only revealed a saturation effect due to the limitation imposed by the light intensity on the rate of quantum absorption ($k_2 = 2600\ s^{-1}$); any $K_m$ is certainly above 20 $\mu$M, and rates corresponding to more than $2 \cdot 10^4$ $\mu$equiv/mg chlorophyll/h could be obtained. When the concentration of oxidised plastocyanin exceeded about 10 $\mu$M, the kinetics became significantly slower, an effect not altered by increasing the concentration of methyl viologen or superoxide dismutase. It is not clear whether this is a sign of formation of a complex between oxidised plastocyanin and P700, or due to some undiagnosed form of cyclic electron flow.

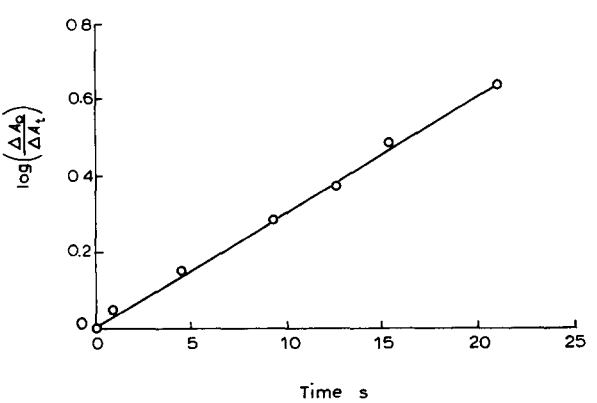


Fig. 2. First-order plot for photo-oxidation of plastocyanin by digitonin treated chloroplasts. The buffer was pH 7.0 phosphate, ionic strength 0.1, and the total volume was 2.42 ml. The reaction mixture contained digitonin at 2 mg/ml, 2.5 $\mu$M plastocyanin (reduced), 100 $\mu$M methyl viologen, 2 $\mu$M DCMU, 50 nM superoxide dismutase, 2 $\mu$l catalase and chloroplasts at 0.41 $\mu$g chlorophyll/ml, giving 0.85 nM P700. After illumination for 30 s, 20 $\mu$l of 7 mM potassium ferricyanide was added to establish the base line for complete plastocyanin oxidation. The temperature was 25.7 °C.

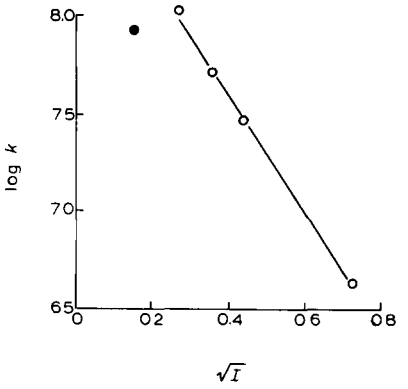


Fig. 3. The effect of ionic strength, $I$, on the rate of photo-oxidation of reduced plastocyanin by digitonin treated chloroplasts. The conditions were generally as in Fig. 2. The point (●) is for 10 mM phosphate buffer, pH 7.0, without added salt. The other points are with varying concentrations of added NaCl.

Fig. 3 shows the effect of increasing ionic strength on the rate coefficient. Brønsted's theory of ionic reactions predicts

$$\log k = \log k_0 + 1.018\, z_A z_B I^{\frac{1}{2}},$$

for a reaction between two species with charges $z_A$ and $z_B$ at 25 °C, where the ionic strength $I = \frac{1}{2}\Sigma m_i z_i^2$, $m_i$ being the molarity and $z_i$ the charge on the ion i. The experimental points for varying concentrations of NaCl lie on a straight line, but the point with no NaCl, only phosphate buffer, lies distinctly below; specific effects of this sort

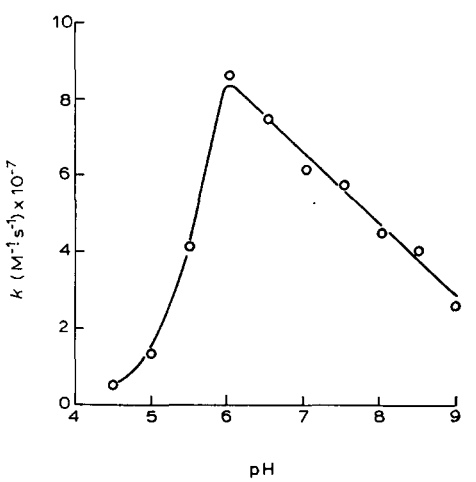


Fig. 4. The pH dependence of the rate of photo-oxidation of reduced plastocyanin by digitonin treated chloroplasts. The conditions were generally as in Fig. 2, with in each case 10 mM buffer and NaCl added to give an ionic strength of 0.1. The buffers used were: succinate for pH 4.5, 5.0, 5.5; MES for pH 6.0, 6.5; HEPES, for pH 7.0, 7.5, 8.0; glycylglycine for pH 8.5, 9.0.

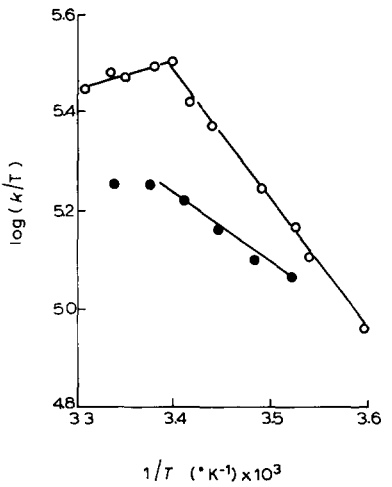


Fig. 5. The temperature dependence of the rate of photo-oxidation of reduced plastocyanin by digitonin (○) and Triton (●) treated chloroplasts, shown as an Eyring plot. The conditions were generally as in Fig. 2. Triton treatment was as described in Methods.

are not uncommon with proteins. The slope of the line corresponds to an interaction between two species of opposite charge, $z_A z_B = -2.9$.

Fig. 4 shows the effect of variation of pH. The rate constant is at a maximum at pH 6.0 and declines sharply as the pH decreases.

Fig. 5 is an Eyring plot of $\log(k/T) : 1/T$ to show the temperature dependence of the rate constant. The lower set of points is for treatment with Triton instead of digitonin. Below 20 °C the points in both cases lie on straight lines; for digitonin the slope indicates an enthalpy of activation $\Delta H^{\ddagger} = 50$ kJ · mol$^{-1}$ (activation energy $E_A = 53$ kJ · mol$^{-1}$; see ref. 1 for a discussion of the formulae involved), whereas for Triton the figures are lower, $\Delta H^{\ddagger} = 27$ kJ · mol$^{-1}$ and $E_A = 29$ kJ · mol$^{-1}$. The points above 20 °C do not lie on the same line, and instead the rate declines slightly with increased temperature, at least for the case of digitonin treatment. There was no sign of any temperature-dependent inactivation at 25 °C.

*Comparison of rates of electron transfer from proteins to P700*

The results described above were all for experiments in the presence of digitonin. Table I shows rate constants for photo-oxidation of plastocyanin by chloroplasts disrupted in three ways: digitonin, Triton and sonication. In each case the listed rate constant is calculated on the basis of the total measured content of P700. The assumption that all of the P700 is exposed to exogenous proteins is almost certainly invalid, but forms a useful basis for comparison and will be discussed further below.

Table I also shows similar data for the other proteins investigated. The rates given in Table I are for the direct reaction

$$X_{red} + P700_{ox} \xrightarrow{k_9} X_{ox} + P700_{red}, \quad (9)$$

where X is the added redox protein. There is also the possibility of an indirect oxida-

TABLE I

ELECTRON TRANSFER FROM PROTEINS TO P700

Rate constants ($M^{-1} \cdot s^{-1}$) are for pH 7.0, ionic strength 0.1 and 25 °C. They are calculated from pseudo-first-order rates ($s^{-1}$) on the basis of all the P700 present being active. The treatment with digitonin, Triton and sonication was as described in Methods.

| | Digitonin | Triton | Sonicated |
|---|---|---|---|
| Plastocyanin | $8 \cdot 10^7$ | $3.6 \cdot 10^7$ | $1.8 \cdot 10^7$ |
| *Plocamium* cytochrome *c*-553 | $1.9 \cdot 10^7$ | $1.3 \cdot 10^7$ | $6 \cdot 10^6$ |
| *Pseudomonas* cytochrome *c*-551 | $8 \cdot 10^6$ | $5 \cdot 10^6$ | $3 \cdot 10^6$ |
| Azurin | $\lessapprox 3 \cdot 10^5$ | $\lessapprox 2 \cdot 10^5$ | $\lessapprox 2 \cdot 10^5$ |
| Cytochrome *f* | $\lessapprox 2 \cdot 10^4$ | $\lessapprox 4 \cdot 10^4$ | $\lessapprox 4 \cdot 10^4$ |
| Horse-heart cytochrome *c* | $\lessapprox 2 \cdot 10^4$ | $\lessapprox 4 \cdot 10^4$ | $\lessapprox 2 \cdot 10^4$ |

tion via endogenous plastocyanin. This is present in the chloroplasts at 6 nmol/mg chlorophyll [6], and after liberation by detergent would give a 2.5 nM solution in a typical experiment with 0.4 μg chlorophyll/ml. The indirect pathway is as follows:

$$PC_{red} + P700_{ox} \xrightarrow{k_1} PC_{ox} + P700_{red} \quad (1)$$

$$X_{red} + PC_{ox} \xrightarrow{k_{10}} X_{ox} + PC_{red} \quad (10)$$

Wood [1] has measured the rate of (10) for all the cytochromes used here. The maximum rate of oxidation of $X_{red}$ by this mechanism is given by $k_1 \cdot [PC][P700]M^{-1} \cdot s^{-1}$. With about 2 μM algal or *Pseudomonas* cytochrome this was negligible compared with the rate of (9) and pseudo-first-order plots similar to Fig. 2 could be used to give the values for $k_9$ shown in Table I. As stated above, the absolute rate constant for photo-oxidation of plastocyanin in the presence of digitonin varied by ±20 % in different chloroplast preparations. By contrast the relative rates with different proteins were essentially constant.

With cytochrome $c_{mam}$ the rate coefficient of the direct reaction, $k_9$, was too slow to measure accurately and the value given in Table I is therefore a limit. From data given above and the rate constant of (10) for cytochrome $c_{mam}$ ($k = 1.0 \cdot 10^6$ $M^{-1} \cdot s^{-1}$; ref. 1) the indirect pathway should saturate at [cytochrome $c_{red}$] $\gtrapprox 0.5$ μM to give a maximal rate of $k_1 \cdot [PC][P700]\ M \cdot s^{-1}$, and this was as observed, with only a very slight increase in rate on adding cytochrome $c_{mam}$ to 10 μM. Oxidation by cytochrome oxidase gave an appreciable dark rate which could be inhibited by cyanide [6, 32, 33] this problem was not found with any other cytochrome.

Cytochrome *f* gave the same behaviour as cytochrome $c_{mam}$ (except for the absence of dark oxidation) and only a limiting value for the rate can be given in Table I. This limit was obtained in experiments in which the endogenous plastocyanin was removed by centrifugation in the presence of digitonin (90 min at 144 000 × *g*), the fragments being resuspended before use.

The rate with azurin, although probably faster, was also too slow for accurate measurement.

*Plastocyanin assay*

Reactions (1) and (10) with added cytochrome $c_{mam}$ formed the basis for Plesničar and Bendall's assay [6] for plastocyanin. It is now clear that a linear depen-

dence of rate of cytochrome $c_{mam}$ oxidation on plastocyanin concentration, as required for the assay, depends on (1) being rate limiting, i.e. [cytochrome $c_{red}$] $\gg$ 100[P700]. The ease and sensitivity of the assay are much improved by use of a dual wavelength instrument as described here, while possible complications can be avoided by addition of superoxide dismutase and catalase.

One somewhat surprising complication which we have found on re-examining the original assay procedure [6] is that addition of KCN (to inhibit cytochrome oxidase) in the absence of added superoxide dismutase can cause a doubling in the rate of photo-oxidation of plastocyanin or cytochrome $c_{mam}$ (in the presence of catalytic amounts of plastocyanin). Similar effects have been reported by Bishop et al. [34] and by Katoh and Takamiya [5]. On the basis of experiments with $O_2^-$ generated by oxidation of xanthine by xanthine oxidase (as in the superoxide dismutase assay) we attribute this effect to small amounts of potassium ferrocyanide, either present in the KCN (BDH Analar KCN is stated to contain 0.02 % Fe) or formed by complexing of iron in the reaction mixture. The reaction of $O_2^-$ with a mixture of ferri- and ferrocyanide leads to incomplete reduction, with a ferricyanide/ferrocyanide equilibrium corresponding to a redox potential of about 0.3–0.35 V, capable of almost completely oxidising cytochrome $c_{mam}$, and partially oxidising reduced plastocyanin ($E_0' = 0.36$ V). It seems that a comparatively fast reduction reaction

$$O_2^- + Fe(CN)_6^{3-} \longrightarrow O_2 + Fe(CN)_6^{4-},\ k \approx 5 \cdot 10^5\ M^{-1} \cdot s^{-1},$$

is balanced by a slower oxidation

$$O_2^- + 2H^+ + Fe(CN)_6^{4-} \longrightarrow H_2O_2 + Fe(CN)_6^{3-},$$

either occurring directly or mediated by a transition metal impurity. Since the $K_m$ for inhibition of cytochrome oxidase by KCN is about 4 $\mu$M [35], while that for superoxide dismutase is about 100 $\mu$M [36, 37], there is no difficulty in inhibiting dark oxidation of cytochrome $c_{mam}$ while still efficiently scavenging $O_2^-$.

## DISCUSSION

### *Comparison of different disruptive methods*

The results presented in Table I show that while the absolute rates (expressed on a total P700 basis) vary for different disruptive methods, there is no change in the general specificity and very little in the relative rates with plastocyanin, red-algal cytochrome *c*-553 and *Pseudomonas* cytochrome *c*-551. The low rates with sonicated chloroplasts probably reflect a low proportion of exposed P700 (sonicated chloroplasts in the presence of digitonin gave the usual high rate). This may also be the case with Triton, in which case the different activation energies with Triton and digitonin may imply a temperature dependence in the proportion of exposed P700.

Our results do not give any evidence that all the P700 is exposed in the case of digitonin treatment, but one can safely say that the absolute rates are not less than those presented here, and the relative rates seem well established. Experiments of this sort are always open to the criticism that the disruption necessary to expose P700 alters the specificity [38], but the very similar relative results with three different disruptive methods, one not employing detergents, and the exceptionally high rate of

reaction with plastocyanin, give hope that our results are not irrelevant to the behaviour of intact thylakoids.

*Plastocyanin+P700*

The rate of this reaction, $k = 8 \cdot 10^7\ M^{-1} \cdot s^{-1}$, is very fast by protein electron transfer standards, being about twice as fast as the rate measured earlier for cytochrome *f*+plastocyanin [1]. But Prince et al. [39] have recently measured an exceptionally fast rate, $k = 8 \cdot 10^8\ M^{-1} \cdot s^{-1}$, for reaction of active centres of *Rhodospirillum* with cytochrome $c_2$; this might be regarded as the analogous reaction in a photosynthetic bacterium.

No value has previously been reported in these dimensions, but the reaction has been studied by many groups, either directly by monitoring plastocyanin [5] or P700 [38], or more often indirectly by the stimulation of oxidation of, for example, ascorbate, reduced dichlorophenolindophenol, or cytochrome $c_{mam}$. Normally a saturation effect has been found at 1–10 $\mu$M plastocyanin [5, 10–13]. Our experiments suggest that this was attributable either to cyclic electron flow, or to a rate limiting step not involving plastocyanin. It is only recently that kinetic measurements for $O_2^-$ and the other species involved have made it possible to rationalise the concentrations of cofactors needed to avoid these problems.

The results on the dependence of the rate on the ionic strength, pH and temperature cannot be related directly to previous work since this all concerns variations in rate under conditions of saturating plastocyanin (at $\lesssim 10\ \mu M$). The inhibition by salt, as found by Nelson and Racker [11], implies an interaction between regions of opposite charge, but this will reflect local charges rather than the total charge on each species [39]. Some earlier values for the optimal pH are 8.0 [40], and 7.0 [13, 41]; the small change in rate from pH 6 to 8 found here means that the precise optimum pH may well be easily changed (e.g. by choice of buffer). The different activation energies obtained with digitonin and Triton show clearly that great caution is needed in applying results of this sort quantitatively to the reactions in intact chloroplasts. The break in both plots at about 20 °C may reflect an effect of temperature on the lipids of the thylakoid membrane.

*Other cuproproteins*

Although azurin has a general resemblance to plastocyanin (both being acidic non-autoxidisable copper proteins with "Type I" spectra showing three peaks at $\lambda > 350$ nm), azurin is not a good donor to P700. This is as found by Anderson and McCarty [42]. Negative results have been reported for some other similar cuproproteins: stellacyanin [40], basic, from *Rhus verniciflua*; mung bean blue protein [10], acidic; and *Anabaena* plastocyanin [13], basic.

*Low molecular weight c-type cytochromes*

Many groups have found soluble *c*-type cytochromes from a wide range of algae to be quite good donors to P700, between two and ten times less efficient than plastocyanin; for examples see refs 10, 43, 44 (*Euglena*), ref. 2 (*Euglena* and *Scenedesmus*) and ref. 45 (*Porphyra* and *Euglena*). These papers and the present results show that while different means of exposing P700 lead to large differences in absolute rates, the relative rates of interaction of P700 with plastocyanin and algal cytochromes vary

much less, suggesting again that the absolute rate may be an indication of the proportion of P700 which is functional in reaction with exogenous proteins.

Our result with *Pseudomonas* cytochrome *c*-551 is the first case known for reaction of higher plant P700 with a protein from a non-photosynthetic organism. This emphasizes the lack of specificity apparently shown by P700 towards low molecular weight, acidic, *c*-type cytochromes.

By contrast, the basic cytochrome $c_{mam}$ is not a good donor to P700 [6, 12]; this was shown by the experiments of Nieman et al. [32] before plastocyanin was discovered. The only exception to the apparent rule that higher plant P700 reacts only with acidic proteins is found in the experiments of Murano and Fujita [12], who reported appreciable activity with the basic cytochrome *c*-553 from *Anabaena*.

*Higher plant cytochrome f*

Cytochrome *f* is an acidic *c*-type cytochrome with a much higher molecular weight than the cytochromes discussed above; for parsley cytochrome *f* (as used here) Forti [46] deduced a molecular weight of 250 000, with four haem groups per molecule, but despite the tetrameric structure electron transfer reactions seem to follow simple kinetics [1]. The absence of measurable direct oxidation of exogenous cytochrome *f* by P700, which we have found both in detergent-treated and sonicated preparations, is in agreement with several other reports for various preparations capable of high rates of photo-oxidation of added plastocyanin [4, 9, 11, 47]. The paper by Kok et al. [10] is often cited as a counter example, but their "cytochrome *f*" was *Euglena* cytochrome *c*-552. Another apparent exception is the moderate rate of cytochrome *f* oxidation reported by Forti and Zanetti [33]; however this was with a preparation containing an appreciable concentration of plastocyanin, similar to that used by Nieman et al. [32] to study cytochrome $c_{mam}$ photo-oxidation.

Despite a few conflicting reports [48, 49] there is increasing evidence for the hypothesis that direct interaction between P700 and endogenous cytochrome *f* is also never fast [50]. Thus the photo-oxidation of endogenous cytochrome *f* is blocked by all the recently discovered inhibitors of plastocyanin: $HgCl_2$ [51], polylysine [52], and KCN [53]. Endogenous cytochrome *f* can be photo-oxidised in some preparations of sub-chloroplast particles, but this is inhibited by adding detergent [38]; it has been shown that such sub-chloroplast particles retain significant amounts of plastocyanin [38, 54, 55], and the residual plastocyanin may well be sufficiently mobile to be not localised to a particular photosynthetic unit [1].

*Comparison of electron transport in higher plants and algae*

The high rates and specificity found by Wood [1] for reactions of purified cytochrome *f* and plastocyanin, and reported here for reaction of plastocyanin, but not cytochrome *f*, with P700 provide additional evidence in favour of a strict order cytochrome *f* → plastocyanin → P700 for oxidation of cytochrome *f* in higher plant photosynthetic electron transport.

There remains an apparent contrast between higher plants, which have a membrane-bound *c*-type cytochrome (cytochrome *f*) evidently not capable of direct interaction with P700, and green algae (which are relatively closely related to higher plants) in which there is a soluble cytochrome *c*, capable of direct electron transfer to P700. Only a limited amount of work has been done on chloroplasts from green algae,

but it is known that *Chlamydomonas* [56] and *Scenedesmus* [57] chloroplasts contain a membrane-bound cytochrome *c*. In *Chlamydomonas*, Gorman and Levine [56] found only 1 molecule of soluble cytochrome *c* per $10^5$ chlorophyll during exponential growth, increasing to 1 per $10^3$ chlorophyll after the cells had entered a stationary phase. Moreover, they found that a mutant *Chlamydomonas* which contained normal amounts of soluble and bound cytochrome *c*-553 but lacked plastocyanin had electron transport reactions of photosystem I blocked; they could be restored by exogenous plastocyanin. They were unable to test adequately whether addition of the soluble form of cytochrome *c*-553 could also effect a restoration, but experiments with *Scenedesmus* plastocyanin and cytochrome *c*-553 interacting with *Scenedesmus* P700 [57] or spinach P700 [2] suggest that such a restoration would have been possible. The simplest interpretation is that the soluble form is not normally involved in photosynthetic electron transport, and that plastocyanin has the same role as in higher plants. There is evidence from mutants of *Scenedesmus* [57] and *Chlamydomonas* [56] that both soluble and bound forms of cytochrome are coded by the same gene; the soluble form may be derived from the bound form by proteolysis, or it may represent molecules not yet incorporated in the electron transport system.

Any extension of these ideas to other classes of algae must remain speculative, but now that plastocyanin has been found in a red alga [58], *Euglena* is the only type studied for which there is no recent report of the presence of plastocyanin. (An absorbance change in *Euglena* at 591 nm was attributed to plastocyanin by Brown et al. [59].) At any rate, our result with *Pseudomonas* cytochrome *c*-551 shows that caution is required in assuming that any cytochrome capable of efficient interaction with P700 has this as its physiological role.

ACKNOWLEDGEMENTS

We should like to thank Dr M. Plesničar for purifying the plastocyanin. P. M. Wood is indebted to Peterhouse, Cambridge for a research fellowship.

REFERENCES

1 Wood, P. M. (1974) Biochim. Biophys. Acta 357, 370–379
2 Hauska, G. A., McCarty, R. E., Berzborn, R. J. and Racker, E. (1971) J. Biol. Chem. 246, 3524–3531
3 Katoh, S. and Takamiya, A. (1965) Biochim. Biophys. Acta 99, 156–160
4 Vernon, L. P. and Shaw, E. (1965) Plant Physiol. 40, 1269–1277
5 Katoh, S. and Takamiya, A. (1963) Plant Cell Physiol. 4, 335–347
6 Plesničar, M. and Bendall, D. S. (1970) Biochim. Biophys. Acta 216, 192–199
7 Brown, J. S. (1972) Carnegie Inst. Year Book 71, 201–205
8 Berg, S., Cipollo, D., Armstrong, B. and Krogmann, D. W. (1973) Biochim. Biophys. Acta 305, 372–383
9 Plesničar, M. and Bendall, D. S. (1973) Eur. J. Biochem. 34, 483–488
10 Kok, B., Rurainski, H. J. and Harmon, E. A. (1964) Plant Physiol. 39, 513–520
11 Nelson, N. and Racker, E. (1972) J. Biol. Chem. 247, 3848–3853
12 Murano, F. and Fujita, Y. (1967) Plant Cell Physiol. 8, 673–682
13 Tsuji, T. and Fujita, Y. (1972) Plant Cell Physiol. 13, 93–99
14 Walker, D. A. (1965) in Biochem. Chloroplasts Proc. Aberystwyth (Goodwin, T. W., ed.), pp. 53–69, Academic Press, New York
15 Arnon, D. I. (1949) Plant Physiol. 24, 1–15

16 Hiyama, T. and Ke, B. (1972) Biochim. Biophys. Acta 267, 160–171
17 McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055
18 Land, E. J. and Swallow, A. J. (1971) Arch. Biochem. Biophys. 145, 365–372
19 Weser, U., Bohnenkamp, W., Cammack, R., Hartmann, H. and Voelcker, G. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1059–1068
20 Klug, D., Rabani, J. and Fridovich, I. (1972) J. Biol. Chem. 247, 4839–4842
21 Hiyama, T. and Ke, B. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1010–1013
22 Witt, K. and Wolff, C. (1970) Z. Naturforsch. B 25, 387–388
23 Hiyama, T. and Ke, B. (1971) Arch. Biochem. Biophys. 147, 99–108
24 Farrington, J. A., Ebert, M., Land, E. J. and Fletcher, K. (1973) Biochim. Biophys. Acta 314, 372–381
25 Beck, G. (1969) Int. J. Radiat. Phys. Chem. 1, 361–371
26 Asada, K., Urano, M. and Takahashi, M. (1973) Eur. J. Biochem. 36, 257–266
27 Saha, S., Ouitrakul, R., Izawa, S. and Good, N. E. (1971) J. Biol. Chem. 246, 3204–3209
28 Nelson, N., Nelson, H. and Racker, E. (1972) Photochem. Photobiol. 16, 481–489
29 Katoh, S. and Takamiya, A. (1961) Nature 189, 665–666
30 Fridovich, I. (1967) J. Biol. Chem. 242, 1445–1449
31 Baxendale, J. H. (1952) Adv. Catal. 4, 31–86
32 Nieman, R. H., Nakamura, H. and Vennesland, B. (1959) Plant Physiol. 34, 262–267
33 Forti, G. and Zanetti, G. (1965) in Currents in Photosynthesis (Thomas, J. B. and Goedheer, J. C., eds), pp. 197–204, Donker, Rotterdam
34 Bishop, N. I., Nakamura, H., Blatt, J. and Vennesland, B. (1959) Plant Physiol. 34, 551–557
35 Yoshikawa, S. and Orii, Y. (1972) J. Biochem. Tokyo 71, 859–872
36 Rotilio, G., Morpurgo, L., Giovagnoli, C., Calabrese, L. and Mondoví, B. (1972) Biochemistry 11, 2187–2192
37 Fried, R., Fried, L. W. and Babin, D. R. (1973) Eur. J. Biochem. 33, 439–445
38 Murata, N. and Fork, D. C. (1973) Carnegie Inst. Year Book 72, 376–384
39 Prince, R. C., Cogdell, R. J. and Crofts, A. R. (1974) Biochim. Biophys. Acta 347, 1–13
40 Katoh, S. and Takamiya, A. (1963) in Photosynthetic Mechanisms of Green Plants, pp. 262–272, National Academy of Sciences, Washington
41 Katoh, S. and San Pietro, A. (1965) in Biochemistry of Copper, pp. 407–422, Academic Press, New York
42 Anderson, M. M. and McCarty, R. E. (1969) Biochim. Biophys. Acta 189, 193–206
43 Vernon, L. P., Ke, B. and Shaw, E. R. (1967) Biochemistry 6, 2210–2220
44 Ke, B. and Shaw, E. R. (1972) Biochim. Biophys. Acta 275, 192–198
45 Fork, D. C. and Murata, N. (1971) Photochem. Photobiol. 13, 33–44
46 Forti, G., Bertolè, M. L. and Zanetti, G. (1965) Biochim. Biophys. Acta 109, 33–40
47 Wessels, J. S. C. (1966) Biochim. Biophys. Acta 126, 581–583
48 Knaff, D. B. (1973) Biochim. Biophys. Acta 292, 186–192
49 Haehnel, W. (1973) Biochim. Biophys. Acta 305, 618–631
50 Siedow, J. N., Curtis, V. A. and San Pietro, A. (1973) Arch. Biochem. Biophys. 158, 889–897
51 Kimimura, M. and Katoh, S. (1972) Biochim. Biophys. Acta 283, 279–292
52 Brand, J., San Pietro, A. and Mayne, B. C. (1972) Arch. Biochem. Biophys. 152, 426–428
53 Izawa, S., Kraayenhof, R., Ruuge, E. K. and Devault, D. (1973) Biochim. Biophys. Acta 314, 328–339
54 Baszynski, T., Brand, J., Krogmann, D. W. and Crane, F. L. (1971) Biochim. Biophys. Acta 234, 537–540
55 Knaff, D. B. and Malkin, R. (1973) Arch. Biochem. Biophys. 159, 555–562
56 Gorman, D. S. and Levine, R. P. (1966) Plant Physiol. 41, 1648–1656
57 Powls, R., Wong, J. and Bishop, N. I. (1969) Biochim. Biophys. Acta 180, 490–499
58 Visser, J. W. M., Amesz, J. and Van Gelder, B. F. (1974) Biochim. Biophys. Acta 333, 279–287
59 Brown, J., Bril, C. and Urbach, W. (1965) Plant Physiol. 40, 1086–1090