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Kinetic studies on redox reactions of *Scenedesmus obliquus* and *Anabaena variabilis* cytochrome *c*-553 with $[Fe(CN)_6]^{3-,4-}$ and $[Co(phen)_3]^{3+,2+}$ and comparisons with data for the corresponding reactions of plastocyanin from the same two algal sources

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Rate constants have been determined over a range of pH for the $[Fe(CN)_6]^{3-}$ (pH 4.0–10.0) and $[Co(phen_3)]^{3+}$ (pH 2.5–9.5) oxidation of ferrocytochrome *c*-553 from *Scenedesmus obliquus* and *Anabaena variabilis*. In spite of the different protein charges (p*I* 4.10 and 8.86, respectively), similar trends in rate constants are observed with both oxidants. A number of protein acid dissociation pK_a values have been determined for these and the corresponding $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ reductions of the ferricytochrome *c*-553's. Comparisons are made with the reactivity of plastocyanin from the same two algal sources, since the respective cytochrome *c*-553 and plastocyanin pairs have similar p*I* values, and are known to function interchangeably in photosynthetic electron transport. Whereas similar rate constant trends are observed for the $[Co(phen)_3]^{3+}$ oxidations of cytochrome *c*-553 and plastocyanin from the same algal source, with $[Fe(CN)_6]^{3-}$ divergent behaviour is observed at the lower pH values. The divergence between $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$, indicated by the ratio k_{Fe}/k_{Co}, is greater for the two positively charged *A. variabilis* proteins. Trends in reduction potentials ($E°$) obtained from rate constants are also compared. Identical $E°$ values are observed at pH 7.5 for the two proteins from *S. obliquus* (380 mV) and from *A. variabilis* (340 mV).

Introduction

The soluble type 1 single Cu protein plastocyanin ($M_r \approx 10\,500$) found in plants and algae functions in photosynthesis as a mobile electron carrier between the membrane-bound cytochrome b_6f complex and P700 [1–3]. In some algae plastocyanin is functionally replaced by a soluble *c*-type cytochrome (variously described as *c*-552, *c*-553 or *c*-554), here referred to as *c*-553 ($M_r \approx 11\,000$; γ-band at 416 nm), which is unknown in higher plants [4,5]. It appears that, in the course of evolution from the first blue-green algae to higher plants, the soluble cytochrome *c*-553 has been replaced by plastocyanin. There is, however, a wide range of green algae and some blue-green algae that contain both the cytochrome and plastocyanin [6]. It appears that both have the same role, and both have been used successfully in the reconstitution of electron transport in *A. variabilis* and *S. obliquus* [7,8]. The equivalent role of the two proteins is reflected by their similar size as well as isoelectric point (p*I*) values [9]. For green algae, p*I* values of cytochrome *c*-553 and

Abbreviations: Mes, 4-morpholineethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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plastocyanin are acidic, whereas for blue-green algae examples of basic as well as acidic proteins are known. It has been noted that the p*I* values vary in a parallel fashion for cytochrome *c*-553 and plastocynanin from one source [9]. It has been established that the role of the cytochrome is quite independent, and different from that of the membrane-bound cytochrome *f* ($M_r \approx 33000$), which has an α-band at 554 nm [10].

In algae which are able to express both plastocyanin and cytochrome *c*-553, the choice between accumulating one or the other protein appears to depend principally upon the availability of Cu in the medium [5,11,12]. If the amount of Cu available is not sufficient to support plastocyanin accumulation in amounts required for photosynthesis the cell uses cytochrome *c*-553 as an alternative electron carrier [13]. There are advantages in being able to interchange Cu with Fe. In a well-oxygenated environment, for example, Cu will be soluble, but iron is liable to be trapped as precipitated ferric hydroxide.

It has been demonstrated that the oxidised forms of plastocyanin and cytochrome *c*-553 from *Chlamydomonas reinhardi* react with cytochrome *f* from the same source, and that the rate of electron transfer from cytochrome *c*-553 to plastocyanin is 25-times slower [5].

Although a full crystal structure of cytochrome *c*-553 has not yet been reported, a crystal structure analysis of the protein from the blue-green algal source *Anacystis nidulans* (using a combination of isomorphous and molecular replacement methods) has established that the same cytochrome-*c*-type fold is present [15]. Also it is clear that the N-terminal, the C-terminal, and the helices incorporating amino-acid residues in the region 40–50 adopt the same conformation relative to the heme as observed in the structure of *Pseudomonas aeruginosa* cytochrome *c*-551 [16]. Differences between *c*-551 and *c*-553 are evident in the loop enfolding the left side of the heme, in the sequence of residues between 18 and 40 on the right side of the heme, and in the region preceding the C-terminal helix. Structural information has also been obtained from ^{1}H-NMR studies [17].

Here we investigate the effect of pH on the reactivity of reduced and oxidised forms of cytochrome *c*-553 with the appropriate partner in the couples $[Fe(CN)_6]^{3-,4-}$ (410 mV) and $[Co(phen)_3]^{3+,2+}$ (370 mV), which have been used extensively as probes for redox activity in studies from this laboratory. Two protein sources have been selected, namely the green alga *Scenedesmus obliquus* [18], in which cytochrome *c*-553 is an acidic protein, and the blue-green alga *Anabaena variabilis* [19], in which it is a basic protein. Comparisons are made with previous data for the reactions of plastocyanin from the same two sources [20,21].

Experimental

Cytochrome c-553 from A. variabilis. The blue-green cyanobacterium *A. variabilis* was grown by a procedure described [22], and the cytochrome purified by the literature method [19], with modifications as here indicated. Frozen cell pastes from five harvests (2 × 20 litre cultures per harvest) were used per work-up. These were thawed and suspended in 0.05 M phosphate buffer (1 litre, pH 7.0). The suspension was sonicated (100 ml portions) at maximum power (using a Rapidis Soniprobe) for 12 min while supported in an ice/water/acetone bath. The sonicated suspension was brought to 45% saturation by the slow addition of solid $(NH_4)_2SO_4$ with continuous stirring over a period of 30 min. Precipitated cell debris and phycoproteins were removed by centrifugation at 10000 rpm for 10 min at 4°C. The supernatant liquid was red-blue, due to the dichroic phycoproteins. Precipitation of the remaining protein was achieved by addition of ammonium sulphate to reach 100% saturation. After standing for 1–2 h the mixture was centrifuged at 10000 rpm for 10 min (4°C). The clear yellow supernatant liquid was decanted with care to avoid disturbing the blue protein pellet. The pellet was taken up in a minimum of 1 mM phosphate buffer at pH 7.0 followed by dialysis against the same buffer. This was then loaded onto a CM-52 column (2.5 × 10 cm), which had been equilibrated with the above buffer (approx. 500 ml) containing a small amount of $[Fe(CN)_6]^{3-}$ to keep the protein fully oxidised. After washing with the same buffer the tightly bound plastocyanin was eluted with 0.05 M buffer at pH 7.0. The slower moving cytochrome *c*-553 band was eluted with 0.2 M

phosphate at pH 7.0. This was purified by passing down a G-50 Sephadex gel column equilibrated with 0.01 M phosphate buffer at pH 7.0. The reduced protein was obtained by addition of sodium dithionite. This gave an absorbance (A) peak ratio of $A_{553}/A_{275} = 0.84 \pm 0.02$, which we used as diagnostic of purity (from the spectrum in Ref. 19a the ratio is 0.86). The absorbance maximum at 553 nm, $\varepsilon = 2.20 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ (see below), was used to determine the concentration of protein.

Cytochrome c-553 from S. obliquus. This green alga was grown [18] and the cytochrome *c*-553 was isolated by the procedures described [23]. Samples of cytochrome *c*-553 were dialysed against 0.005 M Tris-HCl buffer at pH 7.5, loaded onto a DE-52 column (1.5 × 10 cm) equilibrated with the same buffer, and eluted with a linear gradient of 0.005 M (150 ml) to 0.50 M (250 ml) of Tris-HCl buffer at pH 7.5. Protein in its reduced state (reductant sodium dithionite) with peak ratio $A_{553}/A_{275} = 0.75 \pm 0.02$ was used in kinetic studies. Concentrations were determined from the absorbance at 553 nm ($\varepsilon = 2.82 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$).

Complexes. Known peak positions, λ (nm) (absorption coefficients ε ($M^{-1} \cdot cm^{-1}$)) were used in the standardisation/characterisation of complexes. Potassium hexacyanoferrate(II) $K_4[Fe(CN)_6] \cdot 3H_2O$, 330(330), and potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$, 300(1600) and 420(1010) (BDH, AnalaR), were used without further purification. Tris(1,10-phenanthroline)cobalt(III) chloride, $[Co(phen)_3]Cl_2 \cdot 7H_2O$, was prepared and purified to the known spectrum with peak positions 330(4660), 350(3620) and 450(100) [24]. The reductant $[Co(phen)_3]^{2+}$ was generated by adding 1 ml of $CoCl_2 \cdot 6H_2O$ (1 mM) to 25 ml of appropriate buffer solution containing a 6-fold quantity of 1,10-phenanthroline (BDH) ligand. Experiments in which a 12-fold amount of ligand was used gave the same rate constants.

Buffers. The following buffers (10^{-2} M) were used: potassium hydrogen phthalate-HCl (pH 2.5–3.8); sodium acetate/acetic acid (pH 4.0–5.5); Mes/NaOH (pH 5.5–6.5); Hepes/NaOH (pH 6.5–7.5); Tris-HCl (pH 7.2–8.9); sodium tetraborate/NaOH (pH 9.2–10.5). Solution pH values were checked on a Radiometer (PHM 62) pH-meter fitted with a Russell (CWR/322) combined (Ag|AgCl) reference/glass electrode.

Determination of protein coefficients. Analysis of cytochrome *c*-553 samples in the reduced form for Fe were carried out by inductively coupled plasma (ICP) atomic emission spectroscopy. In the case of *S. obliquus* cytochrome *c*-553, a solution with an absorbance at 553 nm of 0.661 was found to have an Fe content of 1.3 μg/ml (i.e., 23.3 μM). The average from three determinations gave values within 5%. Therefore, the absorption coefficient, ε, is $(28.0 \pm 0.7) \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at 553 nm. Similarly, for *A. variabilis*, a solution with absorbance 0.382 at 553 nm gave an Fe content of 1.01 μg/ml (i.e., 17.2 μm) with ε $(22.0 \pm 0.5) \cdot 10^3$ $M^{-1} \cdot cm^{-1}$.

Spectra are illustrated for the oxidised and reduced forms of *S. obliquus* cytochrome *c*-553 in Fig. 1.

Kinetics. All studies were at 25 °C and the ionic strength was adjusted with NaCl to 0.10 M. The cytochrome *c*-553 was reduced with sodium dithionite, and then dialysed against the appropriate buffer under O_2-free conditions. Oxidation of the protein was by adding $[Fe(CN)_6]^{3-}$ before dialysis. In experiments aimed at determining the stoichiometry absorbance changes were consistent with 1 : 1 reactions involving protein in the Fe(II) and Fe(III) states. Inorganic reagents were in at least 10-fold excess of the protein. Runs at pH < 5 were carried out using the pH-jump method. Absorbance changes of the cytochrome *c*-553 at 416 nm were monitored on a Dionex D-110 stopped-flow spectrophotometer, $\Delta\varepsilon = 8.8 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ for *S. obliquus* and $7.6 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ for *A. variabilis*. Plots of $\ln(A_\infty - A_t)$ were linear to 3–4 half-lives and gave first-order rate constants, k_{obs}. In most cases (about 80%), second-order rate constants indicated in figures are an average of two determinations at different concentrations of inorganic complexes.

Determination of reduction potential for A. variabilis cytochrome c-553. A sample of protein was dialysed against 0.10 M phosphate buffer (pH 7.0) after oxidation with $[Fe(CN)_6]^{3-}$. An O_2-free solution of $[Fe(CN)_6]^{4-}$ was prepared ($4 \cdot 10^{-4}$ M) in the same buffer, and small volumes were titrated into 1 ml of protein solution ($5.38 \cdot 10^{-5}$ M). The absorbance was monitored at 553 nm, at which wavelength the cytochrome *c*-553 has ε $(22.0 \pm 0.5) \cdot 10^3$ (reduced) and $(5.0 \pm 0.1) \cdot 10^3$

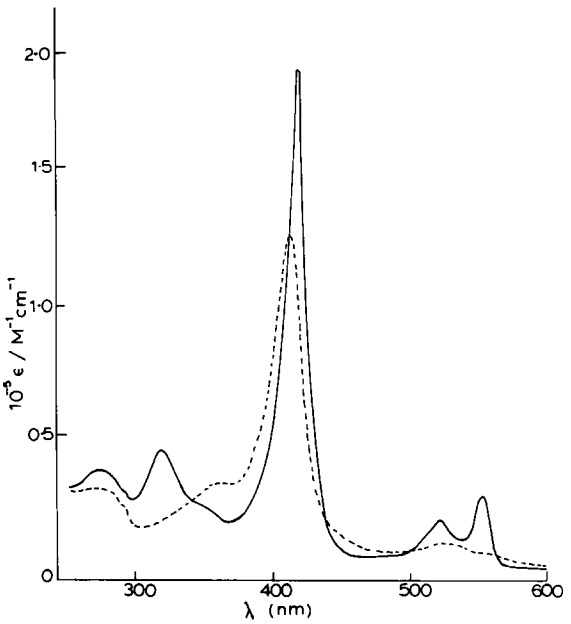

Fig. 1. Ultraviolet-visible spectra of reduced (— — —) and oxidised (———) forms of *S. obliquus* cytochrome *c*-553 at pH 7, $I = 0.10$ M (NaCl).

(oxidised) $M^{-1} \cdot cm^{-1}$. Additions up to 0.2 ml gave a linear plot of [cyt $c_{553}(o)$][$Fe(CN)_6^{3-}$]/[cyt $c_{553}(r)$] against [$Fe(CN)_6^{4-}$], from which the slope gave $K = 0.088$ at 25 °C, and $E°$ of 348 ± 5 mV at pH 7.0, assuming an $E°$ of 410 mV for the $[Fe(CN)_6]^{3-,4-}$ couple.

TABLE I

SEQUENCE HOMOLOGIES FOR *SCENEDESMUS OBLIQUUS* [a] AND *ANABAENA VARIABILIS* [b] CYTOCHROME *c*-553

									10			
S.o.	Ser	Ala	Asp	Met	Ala	Leu	Gly	Lys	Gln	Val	Phe	Glu	Ala
A.v.		Ala	Asp	Ser	val	Asn	Gly	Ala	Lys	Ile	Phe	Ser	Ala
									20			
S.o.	Asn	Cys	Ala	Ala	Cys	His	Ala	Gly	Gly	Asn	Asn		
A.v.	Asn	Cys	Ala	Ser	Cys	His	Ala	Gly	Gly	Lys	Asn		

[a] Information provided by Dr. R.P. Ambler (Edinburgh) (Ambler, R.P. and Powls, R., unpublished data). The full sequence has not yet been determined.

[b] Full sequence available, Ref. 17.

TABLE II

SUMMARY OF PROPERTIES OF *S. OBLIQUUS* AND *A. VARIABILIS* CYTOCHROME *c*-553

	S.o.	*A.v.*
Amino acids	89	90
p*I*	4.10 [a,b]	8.86 [a,c]
No. of aromatic residues	8 [b]	6 [d]
No. of acid residues	< 23 [b]	7 [d]
No. of basic residues	7 [b,e]	12 [d]
Estimated charge	< −16 [a,f]	+5 [a,f]
$E°$ (mV)	380 [b,g]	348 [g]
ϵ ($M^{-1} \cdot cm^{-1}$) at 553 nm	$2.8 \cdot 10^4$ [a,g]	$2.2 \cdot 10^4$ [a,g]

[a] Reduced form.

[b] P. Rowell, Ph.D. Thesis, Department of Biochemistry, University of Liverpool, 1974, p. 38 (Asn and Asp, and Gln and Glu not distinguished).

[c] Ref. 9.

[d] Ref. 17.

[e] Includes one uncoordinated histidine.

[f] At pH ≈ 7.0.

[g] This work.

pK_a for absorbance changes at 695 nm. A solution of *S. obliquus* cytochrome c_{553} ($1.55 \cdot 10^{-4}$ M) in $[Fe(CN)_6]^{3-}$ ($0.50 \cdot 10^{-3}$ M) (to retain the protein in its oxidised form) at $I = 0.10$ M (NaCl), was prepared using an Amicon filtration cell (Model 202) fitted with a PM5 membrane. To 3 ml of this unbuffered solution (pH 6.51), 0.10 M NaOH in 5 μl portions was added and, after allowing 5 min for equilibration at 25 °C, the 650–800 nm spectrum was recorded. From absorbance changes at 695 nm, a pK_a of 8.8 ± 0.1 for *S. obliquus* cytochrome *c*-553 was determined. Using the same procedure, a pK_a of 9.2 ± 0.1 was obtained from horse-heart cytochrome *c* (literature value 9.35) [25]. *A. variabilis* cytochrome *c*-553 also has an absorbance at 695 nm, consistent with methionine coordination.

Amino-acid sequences. The full sequence of *A. variabilis* cytochrome *c*-553 (87 residues) [17] and partial sequence of *S. obliquus* (first 23 residues) [26] are known (Table I). Although belonging to blue-green and green algal types, 14 out of the 23 residues available for comparison are conserved in the two sequences. Only 6 of the first 23 residues are invariant for the twelve sequences in Ref. 23 plus the two considered here. Invariant His-18 and Met-62 residues have been assigned as axial ligands

[17]. Aromatic residues number six for *A. variabilis* and eight for *S. obliquus* compared with nine for horse-heart cytochrome *c* [28]. The charge is estimated as +5 at pH 7.5 on *A. variabilis* cytochrome *c*-553.

Properties of proteins. Relevant properties are summarised in Table II.

Analysis of data. Acid dissociation pK_a values were obtained from the dependence of second-order rate constants on pH using a procedure as in Ref. 29.

Results

For each reaction rate constant, k_{obs} gave a linear dependence on the concentration of inorganic redox partner, and second-order rate constants, k, can be defined by the rate law:

$$\text{rate} = k_{obs}[P] = k[P][C] \quad (1)$$

where P and C denote the protein and inorganic complex, respectively. The variations in k with pH are indicated in Figs. 2–5. Concentrations of reactant C used are indicated in the figure captions. In one case, the reaction of *S. obliquus* cytochrome *c*-553 with $[Co(phen)_3]^{3+}$, where the protein has significant negative charge (p*I* 4.1), the concentration of $[Co(phen)_3]^{3+}$ was varied over a wider range up to $2.5 \cdot 10^{-3}$ M. A plot of k_{obs} vs. $[Co(phen)_3^{3+}]$ remained linear over this range, indicating no significant association of the two reactants prior to electron transfer ($K < 40$ M^{-1}).

The various pH trends observed can be as-

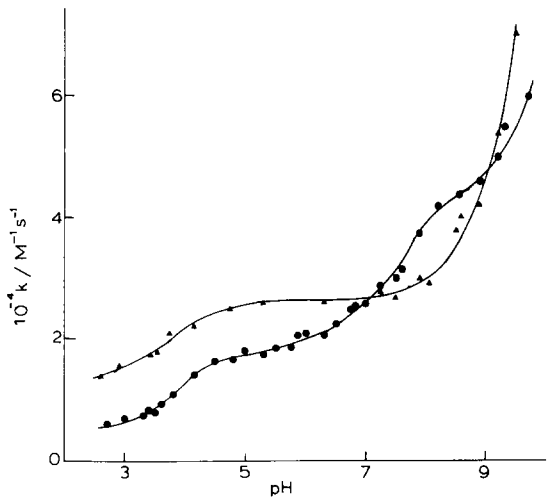

Fig. 3. Second-order rate constants k (25 °C) for the $[Co(phen)_3]^{3+}$ oxidation of ferrocytochrome *c*-553 from *S. obliquus* (●) and *A. variabilis* (▲), $I = 0.10$ M (NaCl). Concentrations of $[Co(phen)_3]^{3+}$ used were in the range $(1–13) \cdot 10^{-5}$ M, and of protein $(1–3) \cdot 10^{-6}$ M.

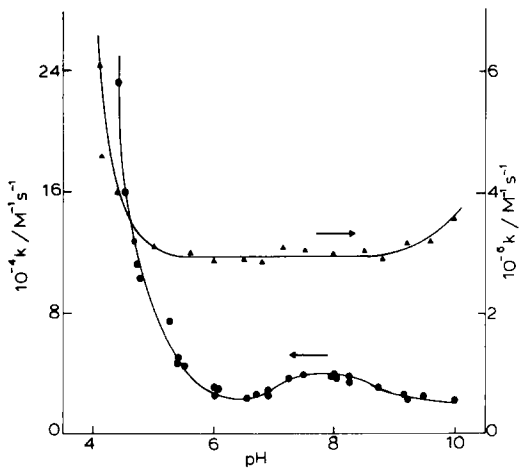

Fig. 2. Second-order rate constants k (25 °C) for the $[Fe(CN)_6]^{3-}$ oxidation of ferrocytochrome *c*-553 from *S. obliquus* (●) and *A. variabilis* (▲), $I = 0.10$ M (NaCl). Concentrations of $[Fe(CN)_6]^{3-}$ used were in the range $(0.5–10.0) \cdot 10^{-5}$ M, and of protein $(1–3) \cdot 10^{-6}$ M.

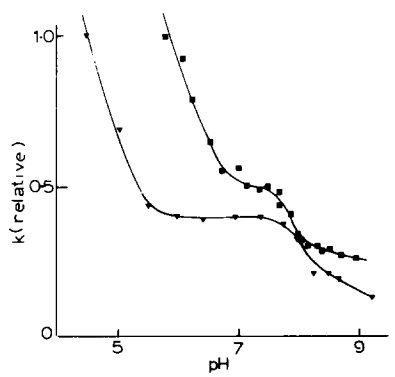

Fig. 4. Second-order rate constants k (25 °C) for the $[Fe(CN)_6]^{4-}$ reduction of ferricytochrome *c*-553 from *S. obliquus* (■) and *A. variabilis* (▼), $I = 0.10$ M (NaCl). Concentrations of $[Fe(CN)_6]^{4-}$ used were in the range $(1.2–3.6) \cdot 10^{-3}$ M (for *S.o.*) and $(1.1–4.1) \cdot 10^{-4}$ M (for *A.v.*).

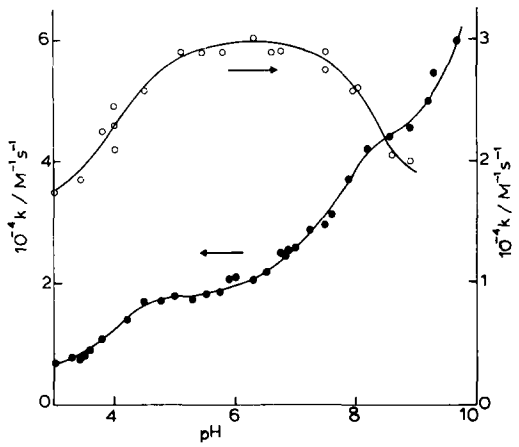

Fig. 5. A comparison of second-order rate constants k (25 °C) for the $[Co(phen)_3]^{2+}$ reduction of ferricytochrome c-553 from *S. obliquus* (○) with data for the $[Co(phen)_3]^{3+}$ oxidation (●) as in Fig. 3, $I = 0.10$ M (NaCl). The concentrations of $[Co(phen)_3]^{2+}$ used were in the range $(0.25–0.5)\cdot 10^{-3}$ M.

signed to effects of protonation/deprotonation of the protein. From information available there is no significant protonation of $[Fe(CN)_6]^{4-}$ at pH > 4.5 (pK_a 4.28 at $I \rightarrow 0$, [30] and 2.33 at $I = 1.0$ M KCl [31]). Protein acid dissociation K_a values, defined as:

$$H^+ P \overset{K_a}{\rightleftharpoons} P + H \qquad (2)$$

were obtained by fitting procedures as in earlier results from this laboratory [20,21] using the expression:

$$k = \frac{k_H[H^+] + K_a k_o}{[H^+] + K_a} \qquad (3)$$

where k_H (protonated) and k_o (unprotonated) are relevant plateau rate constants flanking the relevant pH range. Values of pK_a obtained are listed in Table III. Other details are listed elsewhere [32]. The accuracy of the pK_a values is generally to ± 0.1. In one case, the reaction of *S. obliquus* ferrocytochrome c-553 with $[Co(phen)_3]^{3+}$, a simultaneous two pK_a fitting procedure was necessary to obtain the pK_a values of 6.8 and 8.00 [20,33]. The accuracy of these values is somewhat less at ± 0.25.

Discussion

Cytochrome c-553 and plastocyanin are known to function interchangeably in electron transport from the membrane bound cytochrome b_6f complex to P-700 of Photosystem I in certain green and blue-green algae. The two cytochrome c-553 proteins in this study from *S. obliquus* and *A. variabilis* are acidic and basic, respectively with widely differing pI values of 4.10 and 8.86 (Table II). A comparison of the redox behaviour of the

TABLE III

VALUES OF PROTEIN ACID DISSOCIATION pK_a (25 °C) AS DETERMINED FROM THE VARIATION OF SECOND-ORDER RATE CONSTANTS WITH pH (Figs. 2–5), $I = 0.10$ M (NaCl)

The pK_a values are aligned to indicate similar pK_a values. Arrows indicate trend in rate constants in going from low to high pH for each pK_a. Estimated accuracy ± 0.1 (or less) or as stated.

Reactions of ferrocytochrome c-553	pK_a					
S. obliquus						
$[Fe(CN)_6]^{3-}$	< 4.0 (↓)		7.3 (↑) [a]		8.5 (↓) [a]	
$[Co(phen)_3]^{3+}$	4.1 (↑)	6.8 (↑) [a]		8.0 (↑) [a]		
$[Fe(CN)_6]^{4-}$	< 5.6 (↓)			7.9 (↓)		
$[Co(phen)_3]^{2+}$	4.0 (↑) [a]			8.0 (↓) [c]		
A. variabilis						
$[Fe(CN)_6]^{3-}$	< 4.2 (↓)					
$[Co(phen)_3]^{3+}$	3.7 (↑)					> 9.0 (↑)
$[Fe(CN)_6]^{4-}$	< 5.6 (↓)				8.4 (↓) [b]	

[a] Error ± 0.25.
[b] Error ± 0.3.
[c] Error ± 0.4.

TABLE IV

COMPARISON OF RATE CONSTANTS (25 °C) FOR THE $[Fe(CN)_6]^{3-}$ (k_{Fe}), AND $[Co(phen)_3]^{3+}$ (k_{Co}), OXIDATIONS OF *S. OBLIQUUS* AND *A. VARIABILIS* FERROCYTOCHROME *c*-553 AND PLASTOCYANIN PCu(I) AT pH 7.5, $I = 0.10$ M (NaCl)

Numbers in brackets are for pH 4.5.

	k_{Fe} ($M^{-1} \cdot s^{-1}$)	k_{Co} ($M^{-1} \cdot s^{-1}$)	k_{Fe}/k_{Co}	
S.o. ferrocytochrome *c*-553	$3.8 \cdot 10^4$	$3.2 \cdot 10^4$	1.2	(9.6)
S.o. PCu(I)	$1.0 \cdot 10^5$	$2.0 \cdot 10^3$	50	(205)
A.v. ferrocytochrome *c*-553	$3.0 \cdot 10^6$	$2.8 \cdot 10^4$	107	(170)
A.v. PCu(I)	$6.5 \cdot 10^5$	630	1030	(1291 [a])

[a] At pH 4.77.

two is therefore of interest. Comparisons with the corresponding *S. obliquus* and *A. variabilis* plastocyanins, which have been the subject of previous studies [20,21] and have matching p*I* values of 3.97 and 7.8, respectively, are also of interest.

The two inorganic oxidants chosen for this study have similar reduction potentials (370 and 410 mV), but opposite charges of 3+ and 3−, respectively. The summary of rate constants, Table IV, indicates little difference in reactivity in terms of k_{Fe}/k_{Co} with *S. obliquus* cytochrome *c*-553 at pH 7.5. This behaviour is similar to that observed for three different cytochromes *c*-551 having low charge (in the range +1 to −1) and giving k_{Fe}/k_{Co} values in the range 0.71–1.22 at pH 7.5 [34], but contrasts with that observed for horse-heart cytochrome *c*, where the large number of lysine residues on the front face of the protein, and overall charge (+9 at pH ≈ 7.5), are consistent with $[Fe(CN)_6]^{3-}$ reacting about 10^4-times faster than $[Co(phen)_3]^{3+}$ at pH 7.5 [35]. The point to make is that in the case of the negatively charged *S. obliquus* cytochrome *c*-553 no advantage seems to be taken by the 3+ complex of any negative region of charge, or if it is, the 3− complex is possibly taking equal advantage of a positive locality. No saturation kinetic behaviour was observed with $[Co(phen)_3]^{3+}$ (concentrations up to $2.5 \cdot 10^{-3}$ M), and *K* for prior association of the reactants is less than 40 M^{-1}.

In the case of *A. variabilis* cytochrome c_{553} the oxidant $[Fe(CN)_6]^{3-}$ appears to take advantage of the greater positive charge on the protein, and at all pH levels reacts substantially faster (about 10^2) than $[Co(phen)_3]^{3+}$ (Table IV). Interestingly, a 10^3 discrimination factor also in favour of $[Fe(CN)_6]^{3-}$ is observed with *A. variabilis* plastocyanin. For the PCu(I) state, using ^{1}H-NMR line-broadening techniques, the non-redox active $[Cr(CN)_6]^{3-}$ analogue complex, gave evidence for association in regions of positive charge. The present results for *A. variabilis* cytochrome *c*-553 suggest that a positively charged region, 66–70, may well be influential, and constitute a binding site.

A comparison of amino-acid sequences for cytochrome *c*-553's [27] has indicated a region, 66–76, of high charge density, incorporating both positive and negatively charged residues, Table V. Changes in this region reflect evolutionary aspects, as the overall charge on cytochrome *c*-553 changes from positive to negative. The full sequence for *S. obliquus* cytochrome *c*-553 is not known, but in view of the homologies indicated in Table I it might well also have this highly charged section. It is possible that some of these residues constitute a binding site for reaction with physiological redox partners as well as the complexes used in this study. A necessary requirement for such a binding site is that it should be near to the exposed heme edge.

The variation of rate constants with pH for the reactions of *S. obliquus* and *A. variabilis* cytochrome *c*-553 with $[Fe(CN)_6]^{3-}$ (Fig. 2), and $[Co(phen)_3]^{3+}$ (Fig. 3), are remarkable because, irrespective of overall protein charge, the trends are similar. They are also similar to those observed for cytochrome *c*-551 [34]. At the lower pH levels the trends are consistent with the effect which protonation at carboxylate residues would be expected to have on the electrostatics. As compared to the cytochrome *c*-551's, however, no clearcut assignment of the larger pK_a values (Table III) has emerged. Nor do the further studies with $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ as reductants for ferricytochrome *c*-553 help in making assignments. *S. obliquus* cytochrome *c*-553 has an uncoordinated histidine residue, and this and the heme

TABLE V

A COMPARISON OF AMINO-ACID SEQUENCES, RESIDUES 66 TO 76, FOR CYTOCHROME *c*-553 FROM DIFFERENT BLUE-GREEN ALGAE

The variation in net charge on residues 66–76 (pH > 7.0) parallels the overall charge.

Source	Charge [a] (p*I*)	66	67	68	69	70	71	72	73	74	75	76
Anacystis nidulans [b]	−5 [c] (3.85)	Gly	Ala	*Lys*	Leu	Ser	Ala	**Asp**	**Asp**	Ile	**Glu**	Gly
Synechococcus 6312 [b]	−1	Gly	Gly	*Arg*	Leu	Ser	**Glu**	Ala	Gln	Ile	**Glu**	Asn
Spirulina maxima [b]	0 (5.19)	Asn	Gly	*Arg*	Leu	Ser	Pro	*Lys*	Gln	Ile	**Glu**	**Asp**
Plectonema boryanum [b]	+3	*Lys*	Gly	*Arg*	Leu	Ser	**Asp**	**Asp**	Gln	Ile	Gln	Ser
Anabaena variabilis [d,c]	+5 (8.86)	*Lys*	Gly	*Arg*	Leu	*Lys*	Pro	**Glu**	**Glu**	Ile	Glx	Asx
Aphanizomenon flos-aquae [b]	+9 (9.33)	Gly	*Lys*	*Arg*	Leu	*Lys*	Ala	**Glu**	Gln	Ile	**Glu**	Gly

[a] Estimated total charge on reduced protein, pH > 7.0.
[b] Ref. 9.
[c] Including one uncoordinated histidine.
[d] Ref. 17.

propionates are possible candidates which may give rise to pK_a values of the magnitude observed.

A comparison of rate constant trends with pH for the reactions of cytochrome *c*-553 and the corresponding plastocyanin with $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ as oxidants is illustrated for the *S. obliquus* proteins in Figs. 6 and 7. Rate constants for the $[Fe(CN)_6]^{3-}$ reactions diverge at the lower pH values, the *c*-553 protein becoming increasingly efficient as carboxylate residues protonate, whereas with $[Co(phen)_3]^{3+}$ similar trends are observed, with rate constants becoming very much smaller at the lower pH levels. These contrasting trends are of interest in the context of photosynthesis particularly if the inner thylakoids of the algae are acidic as has been established for the higher plants [36]. At present it is not known whether there are differences in charge also for the physiological redox partners cytochrome *f* and P700 in *S. obliquus* and *A. variabilis*, and how relevant such variations might be.

From the rate constants obtained for both cytochrome *c*-553 with $[Fe(CN)_6]^{3-,4-}$ and $[Co(phen)_3]^{3+,2+}$, it is possible to calculate reduction

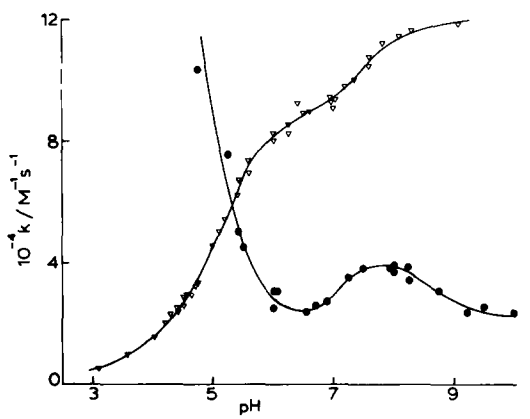

Fig. 6. A comparison of rate constant *k* (25 °C) with pH trends for the $[Fe(CN)_6]^{3-}$ oxidation of *S. obliquus* ferrocytochrome *c*-553 (●), as in Fig. 2, with those observed for the $[Fe(CN)_6]^{3-}$ oxidation of *S. obliquus* plastocyanin PCu(I) (▽, from Ref. 20), $I = 0.10$ M (NaCl).

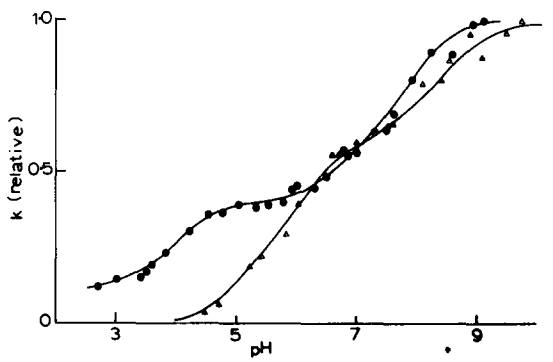

Fig. 7. A comparison of rate constant *k* (25 °C) with pH trends for the $[Co(phen)_3]^{3+}$ oxidation of *S. obliquus* ferrocytochrome *c*-553 (●), as in Fig. 3, with those observed for the $[Co(phen)_3]^{3+}$ oxidation of *S. obliquus* plastocyanin PCu(I) (△, from Ref. 20), $I = 0.10$ M (NaCl).

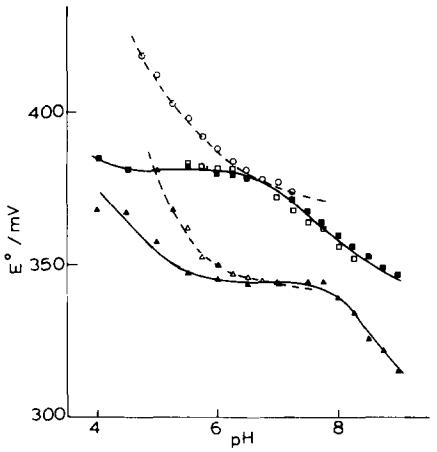

Fig. 8. The variation of reduction potential ($E°$) values with pH as deduced from rate constants for the couples $[Fe(CN)_6]^{3-,4-}$ (410 mV) (open points), and $[Co(phen)_3]^{3+,2+}$ (370 mV) (solid points), *S. obliquus* cytochrome *c*-553 (□, ■), *A. variabilis* cytochrome *c*-553 (▲). Values obtained in a similar way for *S. obliquus* plastocyanin (○, Ref. 20), and *A. variabilis* plastocyanin (△, Ref. 21) are also indicated.

potentials ($E°$) at different pH levels (Fig. 8). The values obtained from a consideration of the two inorganic couples are in excellent agreement, and agree with independently determined values at pH 7.0 (Table II). A comparison with $E°$ values determined in a similar manner for *S. obliquus and A. variabilis* plastocyanin is also made in Fig. 8. It is particularly interesting that at a pH close to 7.0 both the *S. obliquus* proteins have an $E°$ of 380 ± 4 mV, while both *A. variabilis* proteins have an $E°$ of 348 ± 3 mV. The respective trends in $E°$ with pH, although not identical, are very similar.

A final point, from spectrophotometric studies at 695 nm a pK_a of 8.8 was obtained for *S. obliquus* ferricytochrome *c*-553. It has been suggested that the corresponding pK_a for cytochrome *c* arises from the replacement of Met-80 by a lysine residue [37]. In the case of cytochrome *c*-553 a lysine is conserved at position 59 close to the axial ligand Met-62 and may be involved. If such an axial ligand replacement is involved, the $E°$ of the protein will change, and a further pK_a would be expected to manifest itself in all reactions. From NMR, the conserved Tyr-80 has a pK_a of 10.3 ± 0.5 [17], and at higher pH values may also affect the reactivity.

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