Competitive inhibition of electron donation to photosystem 1 by metal-substituted plastocyanin

Hanna Jansson, Örjan Hansson

Department of Chemistry, University of Gothenburg, PO Box 462, SE-405 30 Gothenburg, Sweden

ARTICLE INFO

Article history:
Received 22 October 2007
Received in revised form 24 March 2008
Accepted 27 March 2008
Available online 10 April 2008

Keywords:
Electron transfer
Photosystem 1
Plastocyanin
Redox-induced structural change

ABSTRACT

The electron transfer from wild-type spinach plastocyanin (Pc) to photosystem 1 has been studied by flash-induced absorption changes at 830 nm. The decay kinetics of photo-oxidized P700 are drastically slower in the presence of Ag(I)-substituted Pc, while addition of Zn(II)-substituted Pc has a weaker effect. The metal-substituted forms of Pc act as competitive inhibitors of the reaction between normal, Cu-containing, Pc and P700. The inhibition constants obtained from an analysis of the kinetic data were 30 and 410 μM for Ag(I)- and Zn(II)-substituted Pc, respectively. When the Gly8Asp mutant form of Pc was used instead of the wild-type form, the corresponding values were found to be 77 and 442 μM. If the Ag- and Zn-derivatives can be considered as structural mimics of reduced and oxidized CuPc, respectively, our results imply that there is a redox-induced decrease in the affinity between Pc and photosystem 1 that follows the electron donation to P700. Our data also imply that the Gly8Asp mutation can diminish the magnitude of this change. The findings reported here are consistent with a reaction mechanism where the electron transfer in the complex between Pc and photosystem 1 is assumed to be reversible.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Plastocyanin (Pc) is a small (10.4 kDa), soluble blue copper protein that transfers electrons from cytochrome b6/f to photosystem 1 (PS1) in the photosynthetic electron-transfer (ET) chain. Pc contains a single type 1 copper site, where the Cu ion is liganded by two histidines, one methionine and one cysteine, see Fig. 1. This type of coordination results in a characteristic blue colour for the oxidized protein. See [1] for a recent review of Pc and other type-1 Cu proteins. There are three areas on the protein that are important for the interaction with the redox partners: the hydrophobic, northern end, where the copper is situated, and two acidic patches surrounding Tyr83 on the eastern side. The ET from cytochrome b6/f to Pc [2–6] and from Pc to PS1 [7–14] are both thought to be mediated by the hydrophobic patch via the Cu ligand His87 and the acidic patches probably serve to guide the protein into a proper position for ET. Earlier work in our group has dealt with mutational studies of these patches in order to establish their role in the ET in the Pc–PS1 complex before the ET takes places [15]. In this model the ET is assumed to be irreversible since the reduction potentials of free Pc and P700 are quite different, 384 and 490 mV [10], respectively. However, Drepper et al. [16] found that the reduction potential of Pc shifts to 420 mV upon binding to PS1 and suggested an alternative model where the ET is reversible and no conformational change takes
place in the complex. As shown by Olesen et al. [17], both models account equally well for the observed kinetics, but the interpretation of the observed rate constants in terms of mechanistic rate constants is different in the two models.

If a reversible ET is assumed, then a driving force for ET in the Pc–PS1 complex (Δ$E_p$) of 30–40 mV can be deduced from the kinetic data [17]. This driving force is connected to the difference in reduction potentials and driving force given above, one expects that ET from Pc to P700 weakens the binding to PS1 with a factor of 15–20. This is of course attractive from a physiological perspective, since this would increase the turnover of PS1.

That oxidized Pc (Pc$^{red}$) may bind weaker to PS1 than reduced Pc (Pc$^{ox}$) has been supported by kinetic studies [16] and perturbed angular correlation (PAC) spectroscopy [18]. In the PAC studies, Ag(I)-substituted Pc (AgPc) was used to mimic Pc$^{ox}$ and Cd(II)-substituted Pc (CdPc) was used as a mimic of Pc$^{red}$. The dissociation constant for CdPc from PS1 (with reduced P700) was found to be 24 times larger than for AgPc based on the effect on the rotational-correlation time of the observed rate constants in terms of mechanistic rate constants

\[
\frac{R}{T} \ln \frac{K_{diss}^{obs}}{K_{diss}^{obs}} = \Delta E_p
\]

Here, $K_{diss}$ and $K_{diss}^{obs}$ are the dissociation constants for the Pc–PS1 complex before and after the ET, respectively. Thus, with the reduction potentials and driving force given above, one expects that ET from Pc to P700 weakens the binding to PS1 with a factor of 15–20. This is of course attractive from a physiological perspective, since this would increase the turnover of PS1.

That oxidized Pc (Pc$^{red}$) may bind weaker to PS1 than reduced Pc (Pc$^{ox}$) has been supported by kinetic studies [16] and perturbed angular correlation (PAC) spectroscopy [18]. In the PAC studies, Ag(I)-substituted Pc (AgPc) was used to mimic Pc$^{ox}$ and Cd(II)-substituted Pc (CdPc) was used as a mimic of Pc$^{red}$. The dissociation constant for CdPc from PS1 (with reduced P700) was found to be 24 times larger than for AgPc based on the effect on the rotational-correlation time of Pc on adding PS1 [18].

The dissociation constants $K_{diss}$ and, in particular, $K_{diss}^{obs}$ are difficult to obtain from kinetic data. In the present work we show in a novel way how they can be estimated from the influence of metal-substituted Pc on the flash-induced kinetics of the reaction between normal Pc (CuPc) and PS1. We have used AgPc as a mimic of Cu(I)Pc and Zn(II)-substituted Pc (ZnPc) to mimic Cu(II)Pc. We have found that these redox-inert forms of Pc act as competitive inhibitors of the reaction between CuPc and PS1 but with widely different inhibition constants. Similar studies, using metal-substituted Cu proteins as competitive inhibitors of normal ET reactions, have been made in the past: ET from cytochrome c-550 to amicyanin [19] and from cytochrome $b_6 f$ complex to Pc [20]. However, these studies employed steady-state and stopped-flow kinetics, respectively, rather than the flash-photolysis technique used here and, in both previous cases, the Cu protein (amicyanin and Pc, respectively) acts as an electron acceptor.

The present studies were made both with wild-type spinach Pc, Pc(WT), and with the G8D mutant form of the protein, Pc(G8D) [Fig. 1]. The reason for using the latter is that there are small but significant differences in its reaction with PS1 [13] that need to be further clarified, in particular since crystallographic studies of spinach Pc are made with this mutant and not with Pc(WT), which so far has not been possible to crystallize [21,22].

2. Materials and methods

2.1. Protein purification and metal substitution

Wild-type spinach Pc and the G8D mutant protein were expressed and purified as described by Jansson et al. [23]. PS1 was prepared as described by Sigfriðsson et al. [12].

Ag-substituted Pc was obtained in the following way: Highly purified Pc with an absorption ratio $A_{470}/A_{600}$ of less than 1.2 and at a concentration of approximately 1.7 mM in 10 mM Tris (pH 8.0) was reduced with 5 mM Na-dithionite and desalted on a prepacked PD-10 column. The column contained Sephadex® G-25 (Amersham Pharmacia) pre-equilibrated with 20 mM Hepes (pH 7.5), 0.5 M Na$_2$SO$_4$, 0.1 mM EDTA and 2 mM l-mercaptoethanol. The Pc-containing fractions, as determined by absorbance measurements, were pooled and incubated on ice for 30 min with 50 mM KCN (from a 2.5 M stock solution) to remove the Cu ion. The high ionic strength provided by the Na$_2$SO$_4$ avoids the unfolding of apoPc [24]. The absorption at 597 nm was recorded under oxidizing conditions during the KCN treatment to validate that no Cu was left in the Cu site at the end of the treatment. Cu ions and KCN were removed by a desalting step on another PD-10 column pre-equilibrated with 20 mM Hepes (pH 7.5) and 0.5 M Na$_2$SO$_4$. AgNO$_3$ was added at a tenfold excess over apoPc and then the sample was incubated on ice for 1 h followed by concentration. In order to remove unspecifically bound Ag ions, 7 mM MgSO$_4$ was added and then the sample was desalted again. The whole procedure was performed under reducing and anaerobic conditions with N$_2$-saturated buffers in a glove box to avoid oxidation of the sulphur-containing amino acids in the Cu-site. Chloride-free buffers were used throughout the procedure to avoid precipitates of AgCl.

For production of ZnPc, the protein was expressed and purified as for normal recombinant CuPc [23] except that ZnSO$_4$ was added in the growth media and purification buffers instead of CuSO$_4$.

The success of the metal substitution was verified by nano-spray quadrupole-time of flight (Q-TOF) mass spectrometry performed at the Institute of Biomedicine at University of Gothenburg.

2.2. Flash-photolysis kinetics

The ET from Pc to PS1 was studied by monitoring the absorbance changes due to flash oxidation of P700 in PS1 and subsequent reduction of P700 by Pc. The oxidation of P700 was obtained by a flash from a Nd:YAG laser (Spectra Physics GCR 190-10, wavelength of 532 nm, pulse duration of 8 ns, pulse energy of 5 mJ/cm$^2$). The photooxidation and subsequent reduction of P700 was monitored at 829 nm by a continuous-wave diode laser (Melles-Griot 06DLD203) and a home-built detector (Si photodiode and preamplifier) connected to a digital oscilloscope (Tektronix TDS 3032). PS1 at a concentration of 10 or 6 μM for Pc(WT) and Pc(G8D), respectively, was mixed with buffer, CuPc and Ag or ZnPc in a cuvette with a 1.4 mm optical path length. The buffer consisted of 20 mM Tris (pH 7.5), 7 mM MgCl$_2$, 2 mM sodium ascorbate and 0.1 mM methyl viologen. Sodium ascorbate accelerates CuPc reduction between the flashes and methyl viologen serves as an electron acceptor to PS1. 10–20 different concentrations of CuPc were used, ranging from 20 to 800 μM. Metal-substituted Pc was used at the following concentrations: AgPc at 0.2, 0.5 and 0.5 mM and ZnPc at 0.75, 1.1 mM. For each combination of Pc concentrations, four flash-induced absorption transients were averaged with a spacing of 20 s between the flashes. In addition, the transients were recorded on two different time scales, 40 and 200 ms, with 500 data points for each scale, in order to monitor both fast and slow components in the kinetics.

2.3. Theory

As noted in the Introduction, two different reaction mechanisms have been proposed to account for the multiphasic behaviour of the reduction of P700 by Pc in higher plants.

In the following we will use the model of Drexler et al. [16], which can be written:

\[
Pc^{red} + Ps^{ox} \rightarrow k_{on} \rightarrow k_{fl} \rightarrow k_{off} \rightarrow \text{products}
\]

Here, $k_{on}$, $k_{fl}$, and $k_{off}$ are first-order rate constants (unit: s$^{-1}$) while $k_{on}$ is a second-order rate constant (unit: M$^{-1}$ s$^{-1}$) and red and ox denote reduced and oxidized species, respectively. The analysis by Olesen et al. [17] shows that this reaction model leads to three decay exponents in the reduction kinetics of P700$^{red}$. However, in the limits of...
low and high Pc concentrations, the kinetics will be dominated by two components: a fast phase with a rate constant, \( k_1 \), which is independent of [Pc] and a slow phase with a rate constant, \( k_2 \), which at low [Pc] increases linearly with [Pc] according to

\[
k_2 = k_{20} + k_{21} [\text{Pc}]
\]

(2)

At high [Pc], \( k_s \) saturates at a constant value independent of \( k_{20} \) and \( k_{21} \) [17].

In practice, when the kinetics are followed at 830 nm, it is difficult to observe the expected third phase at intermediate Pc concentrations because there is always a small fourth component due to the formation of Pc** in the reaction. The procedure adopted here is therefore to fit the following triexponential to the experimental absorption transients:

\[
A(t) = A_0 e^{-\frac{t}{\tau_1}} + A_1 e^{-\frac{t}{\tau_2}} + A_2 e^{-\frac{t}{\tau_3}}
\]

(3)

The third, very slow, component accounts for the absorbance of Pcox which decays on a time scale of several ms due to reduction by ascorbate. The corresponding rate constant, \( k_s \), is obtained from data acquired on a longer time scale (200 ms) and this value is then used when fitting Eq. (3) to data on a shorter time scale (40 ms).

The saturating behaviour of \( k_s \) can be modelled by the following hyperbolic function:

\[
k_s = k_{s0} \times \frac{[\text{Pc}]}{C_{\text{Ps}} + [\text{Pc}]}
\]

(4)

Here, \( k_{s0} \) is the saturating value of \( k_s \) at high [Pc] and \( C_{\text{Ps}} \) is the concentration of Pc required for half saturation. The slope at low Pc concentrations can be equated with that of Eq. (2):

\[
k_{s0} = \frac{k_{s0} \times C_{\text{Ps}}}{C_{\text{Ps}} + C_{\text{Ps}}}
\]

(5)

In the experiments reported here, we have studied the influence of Ag- and Zn-substituted Pc on the reaction between CuPc and PS1. These metal-substituted proteins are redox inert but can potentially bind to PS1 and therefore act as competitive inhibitors of the reaction. Their presence is expected to modify \( k_{s0} \) to an apparent value:

\[
k_{s0}^\text{app} = k_{s0} + \frac{K_i}{[\text{Pc}]}
\]

(6)

The inhibition constant \( K_i \) is the equilibrium constant for dissociation of metal-substituted Pc ([Pc]) from PS1. By replacing \( k_{s0} \) with \( k_{s0}^\text{app} \) in Eq. (5) we see that a plot of \( k_{s0}^\text{app} = k_{s0} + \frac{C_{\text{Ps}}}{C_{\text{Ps}} + \frac{[\text{Pc}]^{1}}{K_i}} \) is a straight line, with the slope at a constant value independent of \( [\text{Pc}] \), from which the inhibition constant can be obtained as \( K_i = \frac{[\text{Pc}]}{d} \).

2.4. Curve-fitting and error analysis

The main results of the present work are the inhibition constants \( K_i \) of different variants of metal-substituted Pc. The values are obtained by an analysis of absorption transients signalling the flash-induced ET from Pc to PS1 (Fig. 2) and the uncertainties in \( K_i \) are estimated by a consideration of error propagation as follows.

A sum of two or three decaying exponentials was fitted to the transients using KaleidaGraph 3.6 (Synergy Software). The standard deviations of the \( k_i \) values, as calculated by the software, were of the order of 5–10% of the \( k_i \) values reported in Fig. 3. Slightly higher standard deviations (10–20%) were obtained for the Pc(G8D) data due to a lower concentration of PS1 which resulted in a lower signal-to-noise in the transients. A subsequent fit with KaleidaGraph of hyperbolic functions to the \( k_s \) values in Fig. 3 resulted in values and standard deviations for the parameters \( k_{s0} \) and \( C_{\text{Ps}} \) as reported in Table 1.

The standard deviation in the ratio \( C_{\text{Ps}}/k_{s0} \) was calculated with the following formula for error propagation in a ratio \( x/y \), given the uncertainties \( \sigma_x \) and \( \sigma_y \) in the elements:

\[
\sigma_{x/y} = \sqrt{\left(\frac{x \sigma_y}{y^2}\right)^2 + \left(\frac{x \sigma_x}{y}\right)^2}
\]

(7)

The standard deviations for \( C_{\text{Ps}}/k_{s0} \) are shown as error bars in Fig. 4. Straight lines, \( C_{\text{Ps}}/k_{s0} = C + d[\text{Pc}] \), were then fitted to these weighted data points using a program written in MatLab (MathSoft) and based on procedures described in [25], which yield standard deviations for \( c \) and \( d \). These standard deviations were finally used in Eq. (7) to calculate the standard deviations for \( K_i = c/d \) reported in Table 2.

3. Results

3.1. Protein purification and metal substitution

The Ag- and Zn-substituted Pc required in the present work were obtained by different procedures. ZnPc was produced simply by the replacement of Cu(I) ions with Zn(II) ions in the growth media and purification buffers of recombinant Pc. As judged by absorbance measurements, the purification of ZnPc was successful with as little as 3% CuPc in the final product.

Production of AgPc is more complicated since one has to start with reduced CuPc and all buffers need to be free of chloride ions. Otherwise, addition of Ag ions would result in precipitates of AgCl(s). The procedure described here resulted in a successful substitution of Cu for Ag, with no remaining CuPc in the final product as judged by absorbance measurements.

The AgPc and ZnPc proteins were also analysed with Q-TOF mass spectrometry, which confirmed the successful replacement of Cu with Ag or Zn in the protein. Metal-to-protein ratios of 1.2 and 2.0 were determined for the ZnPc and AgPc proteins, respectively. That the values are larger than one is presumably due to unspecific binding to the protein in addition to binding to the Cu site. No amount of apoprotein could be seen in the mass spectra.

3.2. Flash-photolysis kinetics

Fig. 2 shows the inhibitory effect of AgPc (A) or ZnPc (B) on the ET from CuPc to P700. The transient absorption changes at 830 nm are due to oxidized P700 and, to a minor extent, to oxidized CuPc. A laser flash (vertical arrow) photooxidizes P700 which subsequently becomes reduced by CuPc in a biphasic manner. The reduction is markedly slower in the presence of AgPc (Fig. 2A) while the presence of ZnPc only results in slightly slower kinetics (Fig. 2B) compared with the case of no addition of metal-substituted Pc (Fig. 2C).

The P700** reduction kinetics were measured at several different CuPc concentrations for both the wild-type and the G8D mutant of spinach Pc and in the presence of different amounts of metal-substituted Pc. The resulting absorption transients were subjected to curve-fitting analyses using a model function with several decaying exponentials (see Materials and methods). The two components in the decay of P700** are denoted fast (f) and slow (s). However, a satisfactory curve fit to the data requires an additional, very slow (vs), component in the model function. This third exponential accounts for the absorption of oxidized CuPc that remains at the end of the traces in Fig. 2 and which decays on a time scale of several milliseconds (due to the reduction of oxidized CuPc by ascorbate and to the low-frequency cutoff of the AC amplifier) [12]. Since this
component is unrelated to the reduction of P700$^{ox}$ it will not be further dealt with.

Curve-fitting analyses of the data show that the rate constant of the fast phase ($k_f$) is independent of the CuPc concentration and is not affected by the addition of metal-substituted Pc. Values of $80 \times 10^3$ and $85 \times 10^3$ s$^{-1}$ were found for Pc(WT) and Pc(G8D), respectively, in close agreement with previous findings [13]. The rate constant of the slow phase ($k_s$) displays a characteristic saturating behaviour upon increasing the concentration of CuPc (Fig. 3). The saturating value ($k_{s\text{max}}$) and the CuPc concentration required for half saturation ($C_{ks}$) were obtained from the middle phase in fits of a triexponential function to absorption transients acquired as in Fig. 2. Triangles show the values obtained in the presence of 200 (Δ) or 500 μM Ag-substituted Pc (▲) while diamonds show the values obtained in the presence of 750 (○) or 1.1 mM Zn-substituted Pc (♦). The curves are hyperbolic functions (parameters are given in Table 1) that best fit the data points.

### Table 1

<table>
<thead>
<tr>
<th>Plastocyanin</th>
<th>Inhibitor</th>
<th>Inhibitor concentration (μM)</th>
<th>$C_{ks}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pc(WT)</td>
<td>−</td>
<td>0</td>
<td>43±9</td>
</tr>
<tr>
<td></td>
<td>AgPc</td>
<td>200</td>
<td>454±59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>711±69</td>
</tr>
<tr>
<td></td>
<td>ZnPc</td>
<td>780</td>
<td>125±12</td>
</tr>
<tr>
<td>Pc(G8D)</td>
<td>−</td>
<td>0</td>
<td>101±34</td>
</tr>
<tr>
<td></td>
<td>AgPc</td>
<td>200</td>
<td>450±73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>707±145</td>
</tr>
<tr>
<td></td>
<td>ZnPc</td>
<td>750</td>
<td>234±14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1100</td>
<td>357±22</td>
</tr>
</tbody>
</table>

AgPc and ZnPc denote the Ag- and Zn-substituted forms of spinach Pc. The saturating values of $k_s$ ($k_{s\text{max}}$) and $C_{ks}$ were obtained from fitting a hyperbolic function to the data in Fig. 3. Values of $(14.5\pm0.5) \times 10^3$ s$^{-1}$ and $(23.4\pm2.8) \times 10^3$ s$^{-1}$ were obtained for $k_{s\text{max}}$ in the case of wild-type Pc and the G8D mutant form, respectively, with no inhibitor present. These values were then used in experiments with an inhibitor present. The uncertainties are standard deviations as calculated by the curve-fitting program (see Materials and methods).

![Fig. 3](image-url) Effect of Cu-plastocyanin (CuPc) concentration on the rate constant of the slow phase ($k_s$) in the reduction kinetics of photo-oxidized P700 in the case of wild-type Pc (A, B) or the G8D mutant form (C, D) without any metal-substituted Pc (filled squares, ■). The values were obtained from the middle phase in fits of a triexponential function to absorption transients acquired as in Fig. 2. Triangles show the values obtained in the presence of 200 (Δ) or 500 μM Ag-substituted Pc (▲) while diamonds show the values obtained in the presence of 750 (○) or 1.1 mM Zn-substituted Pc (♦). The curves are hyperbolic functions (parameters are given in Table 1) that best fit the data points.

![Fig. 4](image-url) Effect of metal-substituted plastocyanin (Pc) on the saturating behaviour of the rate constant of the slow phase in the reduction kinetics of photo-oxidized P700 in the case of wild-type Pc (filled symbols) or the G8D mutant form (open symbols). Triangles show the effect of Ag-substituted Pc while diamonds show the effect of Zn-substituted Pc. The parameters $C_{ks}$ and $k_{s\text{max}}$ were obtained from fitting a hyperbolic function to the data in Fig. 3 and the error bars represent the standard deviation of their ratio as calculated by error propagation. The straight lines were fitted to the weighted data points as described in Materials and methods.
from fitting a hyperbolic function, Eq. (4) in Materials and methods, to the data in Fig. 3. The resulting values are listed in Table 1. The parameters are significantly different for Pc(WT) and Pc(G8D), as found previously [13]. Addition of metal-substituted Pc does not affect \( k_{\text{max}} \) but addition of AgPc markedly increases \( C_{\text{eq}} \) while addition of ZnPc only results in a slight increase in \( C_{\text{eq}} \). This behaviour can be explained by a strong competitive inhibition of the ET from CuPc to P700ox by Ag-substituted Pc and a weaker inhibitory effect by ZnPc.

The inhibition constant, \( K_i \), can be obtained from the data in Fig. 3. As outlined in the Theory section in Materials and methods, the ratio \( C_{\text{eq}}/k_{\text{max}} \) is expected to increase linearly with the concentration of metal-substituted Pc. From the straight lines fitted to the weighted data points in Fig. 4 we obtain the \( K_i \) values listed in Table 2. It is clear that ZnPc binds much weaker to PS1 than AgPc. However, there is a difference between Pc(WT) and Pc(G8D): While the Zn-substituted forms have a similar high dissociation constant, AgPc binds 14 times stronger than ZnPc in the case of Pc(WT) while only 6 times stronger in the case of Pc(G8D). The uncertainties in the \( K_i \) values are larger for Pc(G8D) than for Pc(WT) due to the lower concentration of PS1 used for the former (6 \( \mu \)M compared to 10 \( \mu \)M). The larger noise in the absorption transients results in larger uncertainties in the \( K_i \) values which, through error propagation, affects the quality of the final results as explained in Materials and methods.

4. Discussion

The data reported here suggest that AgPc and ZnPc act as competitive inhibitors in the reaction between normal CuPc and PS1. This is as expected, since their structure should not be too different from that of CuPc and therefore they should be able to compete with the latter in its binding to PS1. But, since the metal-substituted forms are redox inert, they will hinder the reduction of photo-oxygenized P700.

The inhibition constants that we find (Table 2) show that AgPc binds stronger than ZnPc to PS1. In the case of Pc(WT), the difference amounts to a factor of 14. This is similar to the finding by Danielsen et al. [18] that AgPc binds 24 times stronger than CdPc to PS1, from PAC-spectroscopic studies. In our kinetic studies we chose ZnPc instead of CdPc because the ionic radius of Zn(II) is much smaller than that of Cu(II). The lack of a suitable isotope of Zn precludes its use in PAC spectroscopy. We have made exploratory kinetic studies of CdPc (prepared in a similar way as AgPc) which indicate that this derivative affects the ET from CuPc to PS1 in a similar way, weak, as ZnPc (data not shown). Thus, if one accepts that Ag(I)-substituted Pc is a good structural mimic of reduced CuPc and that Cd(II)- or Zn(II)-substituted Pc mimics oxidized CuPc, then these data suggest that the reduced form binds 14–24 times stronger to PS1 than the oxidized form.

The observed reduction kinetics of photo-oxygenized P700 can be interpreted in different ways depending on which mechanistic model that is used. Using a model where the ET is assumed to be reversible, Olesen et al. found that reduced Pc binds approximately 20 times stronger to PS1 than reduced Pc in the case of Pc(WT) (Table 5 in [17]). Applying the same calculation procedure on the kinetic data reported here for Pc(WT) without added inhibitor, results in the same value.

This difference in binding strength is similar to the values obtained from the PAC data and from the inhibition studies reported here, but larger than the value of six that Drepper et al. obtained from an analysis of their kinetic data [16].

The findings summarized above are consistent with a reaction mechanism where the ET in the Pc–PS1 complex is assumed to be reversible. The difference of the reduction potentials of free Pc and PS1 is quite large, however (of the order of 100 mV or more, see Introduction). An ET reaction with such a large driving force can hardly be considered as reversible. But, as Eq. (1) shows, if the affinity between Pc and PS1 decreases upon oxidation of Pc, one expects that the driving force for ET is smaller in the complex than for the free molecules. Drepper et al. measured the reduction potentials of Pc and P700 to be 420 and 475 mV, respectively, in a cross-linked complex between the two, which is well in line with the decrease in affinity that they observe [16].

It is interesting to compare these results with other studies where metal-substituted Cu proteins were used as competitive inhibitors of normal ET reactions. Steady-state kinetic studies of the ET from reduced cytochrome c-550 to Cu(II)-amicyanin show that this reaction is strongly inhibited by Zn(II)-substituted amicyanin but not by Ag(I)-amicyanin [19]. Similarly, the ET from reduced cytochrome b(5f) complex to Cu(II)-Pc is severely inhibited by Cd(II)- or Zn(II)-substituted Pc but to a much lower extent by Ag(I)-Pc as shown by stopped-flow studies [20]. Thus, in both these studies the inhibition pattern is opposite to what we report here for the ET from Pc to PS1. However, since the Cu protein acts as an electron acceptor in the previous studies while here it acts as an electron donor, the conclusion is the same: a redox-inert analogue of the product binds weaker to the corresponding reaction partner than that of a reactant analogue.

The data reported here indicate that the reduced state of the Pc(G8D) mutant binds approximately two times weaker to PS1 than the reduced state of the wild-type form (Table 2). Despite the larger uncertainty in the data for Pc(G8D) we consider this to be significant and presumably due to the negative charge of Asp8 located 14.5 Å from the metal ion (Fig. 1) in a position where it can influence the binding to PS1. However, there is still a large difference between the oxidized and reduced states of Pc(G8D) as indicated by the approximately six times larger inhibition constant of the Zn(II)-substituted form of Pc(G8D) compared with the Ag(I)-substituted form (Table 2). Thus, it seems that both Pc(WT) and Pc(G8D) bind stronger to PS1 in the reduced state than in the oxidized state.

A redox-induced change in the affinity between Pc and PS1 could simply be due to a charge effect as suggested by Drepper et al. [16]. However, structural changes may also be important, as suggested in [18]. Indeed, kinetic studies of Pc mutants show that small changes in the hydrophobic patch can drastically change the affinity to PS1. For example, the Pc(L12E) mutant (the location of the mutation is shown in Fig. 1) binds 20 times weaker than Pc(WT) [12] and studies of its structure show that there is only a ca. 1 Å disturbance in the shape of the hydrophobic patch around the mutated residue [23].

We have recently made high-resolution crystallographic studies of the Pc(G8D) mutant in both oxidation states (H. Jansson, M. Ökvist, Ö. Hansson, B.G. Karlsson and L. Sjölin, manuscript in preparation). These studies show that there are redox-induced conformational changes around residue Glu88 in the hydrophobic patch and around residue Gln59 in the small acidic patch. The magnitude of the changes is similar to that induced by the L12E mutation [23]. Thus, we suggest that the difference in binding to PS1 that we report here for AgPc and ZnPc is due to a difference in their structure. This structural difference may mimic a redox-induced structural change in CuPc that influences its affinity to PS1.

Acknowledgments

The work was supported by grants from the Swedish Research Council. We thank Yanis Houssen and Kenneth Olesen for their
contributions at the early stages of this work, Hasse Karlsson for MS analyses and Lars-Erik Andréasson and Lennart Sjölin for helpful discussions.

References