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Redox properties of the *Rhodobacter sphaeroides* transcriptional regulatory proteins PpsR and AppA

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Abstract

Redox properties of the photosynthetic gene repressor PpsR and the blue-light photoreceptor/antirepressor AppA from *Rhodobacter sphaeroides* have been characterized. Redox titrations of PpsR reveal the presence of a two-electron couple, with an E_m value of -320 mV at pH 7.0, which is likely to arise from the reversible conversion of two cysteine thiols to a disulfide. This E_m value is very much more negative than the $E_m = -180$ mV value measured previously at pH 7.0 for the disulfide/dithiol couple in CrtJ, the homolog for PpsR in the closely related bacterium *Rhodobacter capsulatus*. AppA, a flavin-containing blue-light receptor that is also involved in the regulation of gene expression in *R. sphaeroides*, contains multiple cysteines in its C-terminal region, two of which function as a redox-active dithiol/disulfide couple with an E_m value of -325 mV at pH 7.0 in the dark. Titrations of this dithiol/disulfide couple in illuminated samples of AppA indicate that the E_m value of this disulfide/dithiol couple is -315 mV at pH 7.0, identical to the value obtained for AppA in the dark within the combined experimental uncertainties of the two measurements. The E_m values of AppA and PpsR demonstrate that these proteins are thermodynamically capable of electron transfer for their activity as an antirepressor/repressor in *R. sphaeroides*.

Keywords

AppA; CrtJ; Disulfide; PpsR; Redox; Regulation of photosynthetic gene expression; *Rhodobacter sphaeroides*; Transcriptional regulation

Introduction

Expression of genes encoding photosystem proteins in purple non-sulfur photosynthetic bacteria are controlled by a variety of factors including the presence of O₂, light intensity, and light color (Bauer et al. 2003). These regulatory phenomena have been particularly well studied in *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, where the roles of the homologous transcriptional repressors PpsR (*R. sphaeroides*) and CrtJ (*R. capsulatus*) as redox-sensitive DNA-binding proteins have been extensively documented (Bauer et al. 1999, 2003; Bowman et al. 1999; Cho et al. 2004; Elsen et al. 1998; Gomelsky and Kaplan 1997; Kovacs et al. 2005; Masuda and Bauer 2002; Masuda et al. 2002; Oh and Kaplan 2000; Ponnampalam and Bauer 1997; Ponnampalam et al. 1998). While these two bacteria contain only a single *ppsR*

(*crtJ*) gene, other bacteria (e.g., *Rhodopseudomonas palustris* and *Bradyrhizobium* ORS278) contain two *ppsR* genes (Giraud et al. 2002, 2004; Jaubert et al. 2004). Both *R. sphaeroides* PpsR and *R. capsulatus* CrtJ recognize the same palindrome DNA sequence (TGT-N₁₂-ACA) and both have been shown to repress the expression of bacteriochlorophyll (*bch*), carotenoid (*crt*), light harvesting II (*puc*) (Ponnampalam and Bauer 1997), and heme biosynthesis (*hem*) (Smart et al. 2004) genes in response to the presence of O₂. For both *R. capsulatus* CrtJ and *R. sphaeroides* PpsR, the binding affinity of these proteins for their target DNA sequence increases in the presence of O₂ as a result of the oxidation of two cysteine residues that form a disulfide bond (Masuda et al. 2002). Redox-mediated alteration of DNA binding is, however, not a universal feature of this family of proteins since it appears that PpsR2 from *Bradyrhizobium* ORS278 does not respond to changes in cellular redox (Jaubert et al. 2004).

A second protein called AppA has been shown to play a role in controlling gene expression in *R. sphaeroides* in response to both light and O₂ by acting as an antirepressor of PpsR. In regards to redox control, AppA contains a cysteine-rich C-terminal domain that is thought to be involved in the oxidation/reduction of PpsR (Bauer et al. 2003). In addition, AppA has an amino-terminal domain that contains a non-covalently bound FAD (Gomelsky and Klug 2002; Masuda and Bauer 2002). This FAD has been shown to be the chromophore that allows AppA to function as a blue-light photoreceptor (Anderson et al. 2005; Dragnea et al. 2005; Masuda and Bauer 2002; van der Horst et al. 2005). The interaction of AppA with PpsR is light regulated with light excited AppA unable to form an antirepressor–repressor co-complex (Masuda and Bauer 2002). Although the PpsR/CrtJ repressors are widely distributed among photosynthetic bacteria and are highly conserved (see Fig. 1), this is not the case for the antirepressor AppA. For example, this blue-light photoreceptor is not present in either *R. capsulatus* or *Bradyrhizobium* ORS278 (Giraud et al. 2002; Jaubert et al. 2004).

While there is considerable information available about the effect of light absorption by the FAD binding domain of AppA (Anderson et al. 2005; Dragnea et al. 2005; van der Horst et al. 2005) and on the interaction between PpsR and AppA (Bauer et al. 2003), no information available about the oxidation–reduction chemistry of the many cysteine residues in AppA or on the redox-active Cys in PpsR. For *R. capsulatus* CrtJ, E_m values of –180 mV at pH 7.0 and of –245 mV at pH 8.0 have been measured for the redox-active Cys249/Cys420 disulfide/dithiol couple (Masuda et al. 2002). The ambient redox potential in the cytoplasm of anaerobic cultures of *R. capsulatus* has been estimated to be –220 mV determined by the GSH/GSSG ratio (Masuda et al. 2002). If the internal pH in *R. capsulatus* cells is close to 7.0, CrtJ would thus be expected to be predominantly reduced in anaerobic cells if the protein is in redox equilibrium with its cellular environment, a prediction that has been experimentally verified (Masuda et al. 2002). It has also been shown that CrtJ is predominantly oxidized in aerobic cultures of *R. capsulatus*, even though the presence of O₂ has no discernable effect on the internal ambient potential of the cells (Masuda et al. 2002). It has thus been concluded that in *R. capsulatus* CrtJ functions as an O₂-sensor rather than as a sensor of ambient potential and that it is not always at redox equilibrium with the cellular potential (Masuda et al. 2002). Below we report E_m values and quantitative evaluations of the thiol contents for both AppA and PpsR from *R. sphaeroides*, and also examine the effect of light on the redox chemistry of AppA. The data obtained in this study provide the first framework for examining the thermodynamic favorability of the reduction of PpsR by AppA, a reaction that has been demonstrated in vitro studies of the two purified *R. sphaeroides* proteins (Masuda et al. 2002).

Materials and methods

Protein overexpression and purification

Rhodobacter sphaeroides AppA was purified according to published protocols (Masuda and Bauer 2002). A His-tagged version of the *R. sphaeroides* PpsR protein was expressed in

Escherichia coli strain BL21(DE3) (Novagen). The bacteria were grown in Luria Broth (LB) medium containing 20 g/l Tryptone, 10 g/l yeast extract, 20 g/l NaCl, and 50 mg/l kanamycin. The cultures were induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) grown for 4 h at room temperature with shaking. Cells were then harvested by centrifuging for 10 min using a Beckman JA-10 rotor at $6000 \times g$. The cells were then resuspended in buffer containing 50 mM HEPES pH 7.0 and 100 mM NaCl. Cells were lysed by two passages through a French press at 8000 psi. The resulting solution was centrifuged at $28,000 \times g$ for 30 min to pellet out the cell debris. The supernatant was then centrifuged for 1 h at $100,000 \times g$ in a Ti-70 rotor. Finally, the supernatant was passed through a 0.45 micron filter and then loaded onto a Ni^{2+} -charged chelating column (Hi-Trap by Pharmacia). After washing the column to remove nonspecifically bound proteins, the remaining protein was eluted with buffer containing 250 mM imidazole. The eluate was then dialyzed overnight against a buffer containing 30 mM HEPES (pH = 7.0) and 100 mM NaCl. Protein purity was subsequently determined by SDS-PAGE gel electrophoresis to be >95% pure. Samples with purity < 95% were loaded onto a Superdex 75 column and remaining peaks were separated out, collected in fractions, and re-concentrated to volumes of 1–2 ml.

Redox titrations

To obtain PpsR samples with the maximum disulfide content, the protein was incubated with oxidized dithiothreitol (DTT), while PpsR samples with maximum reduced thiol content were obtained by incubating the protein with reduced DTT. The DTT was subsequently removed by gel filtration on a Sephadex G-10 column. Total free thiols were titrated with 5,5'-dithiolbis (2-nitrobenzoic acid) (DTNB) in 100 mM Tris-HCl, pH 8.0 (Balmer et al. 2001). Release of the chromophore 5-thiol-2-nitrobenzoic acid (TNB) was monitored at 412 nm, using a Shimadzu spectrophotometer (Model UV2401 PC) at 1.0 nm spectral resolution, and its concentration was calculated using a molar absorptivity of $13600 \text{ M}^{-1} \text{ cm}^{-1}$.

Oxidation–reduction titrations were carried out as described previously, using either the fluorescence of the monobromobimane (mBBR) adducts of the proteins or the intrinsic tryptophan fluorescence of the proteins to monitor the presence of thiols (Hutchison and Ort 1995; Setterdahl et al. 2003). Fluorescence was measured using an Aminco-Bowman Series 2 Luminescent Spectrometer. An excitation wavelength of 380 nm and an emission wavelength of 450 nm were used for the mBBR measurements and an excitation wavelength of 275 nm and an emission wavelength of 337 nm was used for the tryptophan fluorescence measurements (both emission and excitation monochromators were operated at a spectral resolution of 1.0 nm). Redox equilibration was carried out in buffers containing defined ratios of oxidized to reduced DTT, as described previously (Setterdahl et al. 2003) (Preliminary measurements, carried out over a more positive range of ambient potentials, used mixtures of reduced and oxidized glutathione to establish defined potentials, were discontinued after it was found that no PpsR or AppA disulfide/dithiol redox couples titrated in this range.). Essentially identical titration curves were obtained, independent of the redox-equilibration time spanning from 1 to 4 h and of the total DTT concentration over the range from 1 mM to 5 mM present in the redox-equilibration buffer. Essentially identical titration curves were obtained regardless of whether the titrations were carried out under ambient atmosphere or under an oxygen-free argon atmosphere. For the AppA “dark titrations,” the samples were covered with black cloth during the redox-equilibration period. For the AppA “light titrations” the samples were illuminated with white light at an intensity of 7,000 lux measured with a VWR Scientific light meter throughout the entire redox-equilibration period. In order to prevent protein denaturation from heating caused by the illumination, the samples were kept at 5°C during illumination.

Titration data were fitted to the Nernst equation for a single two-electron ($n = 2$) redox process occurring at 20°C with the Nernst equation used to fit the data for the illuminated samples

modified to take into account the fact that these titrations were carried out at 5°C. No improvement in the fit was obtained using a two-component version of the Nernst equation. Experimental uncertainties are reported as average deviations due to the limited amount of sample available. The pK_a value of the redox-linked acid/base present in AppA was calculated, as described previously (Setterdahl et al. 2003), by fitting the E_m vs. pH data for AppA to the following equation:

$$E_m = E^\circ - (RT/nF) \log \{ 1 / ([H^+]^2 + [H^+]K_{a1} + [H^+]K_{a2} + K_{a1}K_{a2}) \} \quad (1)$$

where E° represents the intrinsic tendency of the disulfide bond to undergo reduction, and K_{a1} and K_{a2} are the first and second K_a values for the acid dissociation constants for the proton equilibrium coupled to the redox reaction. E° was set so that the equation gave an E_m value at pH 7.0 that matched the experimentally determined value, while K_{a1} and K_{a2} were determined as best-fit parameters using chi-squared error in MicroSoft Excel. Over the range of pH values where AppA was stable enough to carry out redox titrations successfully, only a single pK_a value was detected.

Results

Redox titration of PpsR

Based on a high degree of amino acid sequence homology between *R. sphaeroides* PpsR and *R. capsulatus* CrtJ (see Fig. 1) we initially assumed that PpsR would have a similar E_m as previously reported for CrtJ (Masuda et al. 2002). However, no changes in the fluorescence levels of mBBr-labeled PpsR could be detected, as the ambient redox potential (E_h) was varied over the full range of potentials (−100 to −260 mV at pH 7.0) accessible with GSH/GSSG redox buffers. We also observed that incubation of oxidized PpsR with GSH did not lead to reduction of oxidized PpsR (data not shown), which is contrasted by the ability of GSH to reduce CrtJ (Masuda et al. 2002). We therefore altered the redox titration to use DTT as a reductant since it is capable of forming disulfide/dithiol couples over a more negative E_h range. Figure 2A shows the results of an mBBr redox titration of PpsR carried out at pH 7.0 with an ambient temperature of 20°C. The data gave a good fit to the $n = 2$ Nernst equation for a single component with an $E_m = -320$ mV. The average E_m value for PpsR at pH 7.0, from a series of five titrations, was -320 ± 10 mV which is 140 mV more negative than the −180 mV E_m value previously determined for *R. capsulatus* CrtJ (Masuda et al. 2002).

Tryptophan fluorescence emission spectra, obtained by excitation of fully oxidized and fully reduced PpsR at 275 nm (not shown), indicated that the magnitude of intrinsic tryptophan fluorescence exhibited by the protein is also a function of the dithiol/disulfide oxidation state of the protein. This phenomenon allowed independent redox titrations to be carried out using tryptophan fluorescence to monitor dithiol content. The tryptophan fluorescence titrations of PpsR at pH 7.0 also gave excellent fits to the $n = 2$ Nernst equation (not shown), with an average E_m value of -340 ± 5 mV (two titrations). The difference in average E_m values obtained by the two different monitoring methods is approximately the same as the sum of the experimental uncertainties in the two sets of measurements and thus is unlikely to be significant, but may also represent a small perturbation of the redox system caused by the addition of mBBr.

Table 1 shows the results of DTNB measurements of the number of thiols present at positive and negative E_h extremes of the titration shown in Fig. 2A. The data indicate that *R. sphaeroides* PpsR treated with oxidized DTT contains no DTNB detectable thiols, while PpsR treated with reduced DTT contains, within experimental uncertainties of the measurement, two thiols. As this protein contains only two cysteine residues (see Fig. 1), the most likely

conclusion is that the oxidized form of PpsR contains a single intramolecular disulfide and that the reduced form contains two cysteine thiols. Another possibility is that the disulfide formed at the most positive E_h values shown in Fig. 2A, is an intermolecular disulfide linking two PpsR molecules. In this case, the oxidized form of the protein would contain only one DNTB accessible thiol per monomer with a second thiol buried in the interior of the protein and inaccessible to DTNB. This second possibility cannot be unambiguously eliminated, but the simplest explanation for the data of Table 1 is that the oxidized form of PpsR contains an intramolecular disulfide.

The results of measuring the E_m value of PpsR over a range of pH values where PpsR remains stable are summarized in Fig. 2B. The open circles, with ± 10 mV error brackets added, represent the average values of at least two replicate titrations at each pH value. The solid line is the straight line that has a best-fit slope of -55 mV/pH, which is within the combined experimental uncertainties involved in the measurements. Note that the theoretical expected for a reaction in which two protons are taken up by the protein per disulfide reduced is -59 mV/pH unit slope (Hutchison and Ort 1995; Setterdahl et al. 2003). The obtained experimental value is within experimental uncertainty of the theoretical value suggesting that there are no acid/base groups in the protein with pK_a values between 5.5 and 10.0, approximately. This indicates that the pK_a values for both of the cysteines in reduced PpsR must lie above 9.5, and that the two protons taken up on reduction are used to protonate the cysteines formed on reduction, producing the thiol forms of both cysteines rather than the unprotonated thiolate anions at all pH values below 9.5. However, it should be noted that thermodynamic considerations used to predict the slope of a E_m vs. pH profile simply allow the number of protons taken up to be measured but cannot provide any information as to the specific sites of proton uptake.

Redox titration of AppA

Similar redox titrations were performed with the redox-active antirepressor, AppA. For this analysis we were only able to assay mBBr titrations given that the magnitude of AppA's intrinsic tryptophan fluorescence was not affected by the disulfide/dithiol redox state of the protein. Thus, it was not possible to carry out redox titrations of AppA by measuring changes in tryptophan fluorescence. The open circles in Fig. 3A represent data points from a redox titration of AppA in the dark at pH 7.0 and at ambient temperature, using mBBr labeling of the protein to monitor its thiol content. The mBBr titrations of Fig. 3A were carried out under the same conditions used for the mBBr titration of PpsR shown in Fig. 2A in order to maximize the reliability of comparisons between the E_m values of PpsR and AppA. As shown by the solid line, the data gave a good fit to the $n = 2$ Nernst equation for a single component with $E_m = -320$ mV. An average E_m value of -325 ± 10 mV was obtained from three replicate titrations carried out at ambient temperature using dark-adapted samples at pH 7.0.

The closed circles shown in Fig. 3A represent data points from an mBBr titration of AppA at pH 7.0, using a sample in which the redox-equilibration stage was carried out at 5°C under continuous illumination with white light at an intensity of 7,000 lux. As can be seen from the dashed line, the data from light excited AppA also gave a good fit to the $n = 2$ Nernst equation for a single component with $E_m = -315 \pm 10$ mV average obtained from three replicate titrations of illuminated samples. This value is within the combined experimental uncertainties of the measurements of the value obtained for AppA samples maintained in the dark.

Titrations on illuminated samples were carried out at 5°C in order to prevent protein denaturation during the protracted illumination period. In order to make a direct comparison of light and dark E_m values for AppA, it requires that E_m of dark-adapted AppA also be undertaken at 5°C . Analysis of the E_m value for AppA in the dark at pH 7.0 and 5°C was identical to the -325 mV value obtained at ambient temperature (data not shown), indicating

that, at least over the temperature range from approximately 5–20°C, the E_m of AppA is unaffected by temperature. It can thus be concluded that illumination of AppA does not produce a statistically significant change in the E_m value of the one redox-active disulfide present in the protein. It is also worth noting that, within the combined experimental uncertainties of the measurements, the disulfide/dithiol couples in AppA and PpsR have nearly identical E_m values at pH 7.0.

The possibility that the component being titrated in AppA is a redox-active disulfide/dithiol couple is supported by the data of Table 1, which show that reduction of oxidized AppA results in an increase in the number of DTNB-reactive thiols of 2.5 ± 0.9 , which is within the experimental uncertainty of the value of two expected for reduction of an intramolecular disulfide. The total number of DTNB-reactive thiols observed for reduced AppA is 8.4 ± 0.5 , two to three less than the value of 11.0 that would be expected if all 11 cysteine residues in AppA were accessible to DTNB. The simplest interpretation of this data is that two to three of the 11 AppA cysteines are buried in the interior of the protein or possibly that one Cys is buried and inaccessible to DNTB and that the other two Cys form a disulfide that is not reduced by DTT.

Fig. 3B shows the E_m vs. pH profile for AppA, titrated in the dark at ambient temperature, over the pH range from 5.5 to 8.5 (the protein was not sufficiently stable at alkaline pH to obtain reliable E_m values at pH values above 8.5). Unlike the case for PpsR (see Fig. 2B), the data cannot be fitted using a single straight line with a slope of -59 mV/pH unit. The data do give a good fit to two intersecting straight lines, one (in the more acidic region) with a slope of -59 mV/pH unit and a second (in the more alkaline region) with a slope of -29.5 mV/pH unit that is characteristic of a transition (in the region around the pH = 7.5 intersection of the two lines) from the uptake of two protons per disulfide reduced to the uptake of one proton per disulfide reduced. The E_m vs. pH data of Fig. 3B also gave an excellent fit to Eq. 1 (not shown) with a calculated K_{a1} corresponding to a pK_a value of 7.5 for the redox-coupled acid/base present in AppA. It was not possible to calculate K_{a2} from the data of Fig. 3B but it can be estimated that no other redox-linked pK_a value below 9.0 is present. While the simplest interpretation of this data is that the thiol group of one of AppA's redox-active cysteines has a pK_a of 7.5, it must be reiterated that these equilibrium thermodynamic measurements cannot, by themselves, identify the chemical nature of the group responsible for the redox-linked pK_a . It should also be mentioned that, because AppA and PpsR exhibit identical E_m vs. pH behaviors between pH 5.5 and a pH of approximately 7.2, the disulfide/dithiol couples of the two proteins will be isopotential over this pH range but that the E_m value of PpsR will become increasingly more negative than that of AppA as the pH increases above 7.2.

Discussion

An interesting aspect of this work is the finding that the E_m value of the redox-active disulfide/dithiol couple in *R. sphaeroides* PpsR is so much more negative than that measured previously (Masuda et al. 2002) for the corresponding couple in *R. capsulatus* CrtJ. Given that PpsR and CrtJ are 53% identical at the amino acid level, the difference of 140 mV in the E_m values of these two intramolecular disulfide bonds is quite surprising. One can only conclude that the microenvironments of the two redox-active cysteine pairs (i.e., Cys251 and Cys424 in *R. sphaeroides* PpsR and Cys249 and Cys420 in *R. capsulatus* CrtJ) must be different. The amino acid sequences of the C-terminus that include the helix-turn-helix DNA binding domain and Cys424 (using the *R. sphaeroides* PpsR numbering), are more highly conserved among members of the PpsR/CrtJ family than is the region containing the less well-conserved Cys249 (see Fig. 1). Single amino acid substitutions near redox-active site cysteines can alter the E_m value of disulfides significantly (Setterdahl et al. 2003). Thus, it may well be that differences in the Cys249 region of these proteins are largely responsible for the observed variability in

E_m value. However, in the absence of three-dimensional structures for the two proteins, assigning a structural basis for difference in redox properties remains speculative. Furthermore, PpsR1 from *Bradyrhizobium* ORS278 has only one cysteine (Cys429, see Fig. 1) and yet it can regulate gene expression in a redox-dependent manner, possibly involving a PpsR1 homodimer linked through a disulfide involving one Cys429 from each monomer as has been clearly shown by Jaubert et al. (2004) by non-reducing SDS-PAGE and gel filtration experiments, though it remains possible for a mixed disulfide with GSH or other cysteine oxidizing molecules in vivo. Thus, the differential DNA-binding properties of some members of this family of regulatory proteins may involve redox-active intermolecular disulfides, in contrast to the intramolecular disulfides found in the *R. sphaeroides* PpsR and *R. capsulatus* CrtJ.

A series of in vitro experiments have been carried out to elucidate the possible roles of *R. sphaeroides* AppA and PpsR in the ability of this bacterium to regulate gene expression in response to light and O_2 (Masuda and Bauer 2002). Oxidized PpsR binds its target DNA sequence very tightly, but the binding affinity of PpsR to its target DNA is greatly diminished when reduced AppA is mixed with oxidized PpsR in the dark. AMS modification experiments have demonstrated that reduced AppA is indeed capable of reducing oxidized PpsR in vitro (Masuda and Bauer 2002). However, the ability of AppA to reduce oxidized PpsR is inhibited if the reaction is carried out under illuminated conditions (Masuda and Bauer 2002). Indeed, subsequent experimentation has demonstrated that AppA is a flavin containing blue light absorbing photoreceptor that undergoes a conformational change upon light absorption by FAD. Under conditions of light excitation, AppA is unable to form a tight complex with PpsR (Masuda and Bauer 2002). It has thus been proposed that AppA-mediated antirepression in *R. sphaeroides* involves two stages (i) reduction of a disulfide bond in oxidized PpsR by the reduced dithiol form of AppA and (ii) dark formation of an AppA-(PpsR)₂ complex that interferes with the DNA-binding activity of PpsR (Masuda and Bauer 2002).

Although the proposed mechanism of AppA-mediated antirepression in *R. sphaeroides* requires that AppA be able to reduce oxidized PpsR, no data was available on the oxidation–reduction properties of either PpsR or AppA from *R. sphaeroides*. Thus, although it had been demonstrated that purified, reduced AppA can reduce purified PpsR in vitro, the thermodynamic basis for this transfer of reducing equivalents was not known. To fill in this gap in our understanding of these two important regulatory proteins, we have carried out oxidation–reduction titrations of both proteins. Rather than carrying out these measurements at only pH 7.0, we have measured E_m values for both proteins over a wide range of pH values. Knowledge of these E_m vs. pH profiles not only allows one to calculate the thermodynamic driving force for electron transfer at any pH value likely to occur in the cytoplasm of *R. sphaeroides* cells, but also allows one to detect the presence of acid/base groups in the protein with pK_a values that depend on the redox state of the protein. Our observation that the two proteins are isopotential at pH 7.0 allows us to predict that the reduction of PpsR by AppA is a reaction with an equilibrium constant of 1.0. This calculation applies only to redox reactions that reach thermodynamic equilibrium and, as we have previously demonstrated for the behavior of *R. capsulatus* CrtJ, redox-active groups on these regulatory DNA-binding proteins, are in fact not always in redox equilibrium with their cellular environment (Masuda et al. 2002). This also appears likely to be the case for PpsR and AppA in *R. sphaeroides*. Although the ambient internal redox potential of *R. sphaeroides* is not known, it has been estimated to be approximately -220 mV in the closely related *R. capsulatus*. At an E_h value of -220 mV, where the ambient potential is 100 mV more positive than the E_m value of both PpsR and AppA, both proteins would be more than 99.9% oxidized at equilibrium, making the extent of electron transfer between the proteins negligible. It thus seems highly probable that one or both of the proteins is not in redox equilibrium with the cellular ambient potential, at least for significant periods of time.

If one assumes that electron transfer between AppA and PpsR is in fact a reaction that reaches equilibrium, a reaction in *R. sphaeroides* between fully reduced AppA and fully oxidized PpsR will result in a substantial reduction of PpsR in a thermodynamically favorable manner. The fact that the E_m vs. pH profiles of the two proteins exhibit the same slope in the region from pH 5.5 to approximately pH 7.2 means that the equilibrium constant for PpsR reduction by AppA will be independent of pH in this region. However, as the pH increases above approximately 7.2, the E_m value of PpsR will become increasingly more negative than that of AppA and reduction of PpsR by AppA will become increasingly more unfavorable. It should also be mentioned that protein–protein interactions within a protein/protein complex can shift the E_m value of one or both of the proteins and thus the thermodynamic driving force for the transfer of reducing equivalents between AppA and PpsR may differ from that predicted from E_m values measured for the isolated proteins. If complex formation does produce such shifts in E_m values, then light—which causes conformational changes that lead to dissociation of the complex—could affect the reduction of PpsR by AppA.

The transcriptional regulator PpsR has been identified in all purple bacteria, yet different species of these bacteria utilize these proteins in different ways, even when regulating the same set of genes. One example is PpsR1 from *Bradyrhizobium* ORS278 that regulates gene expression in response to redox-related stimuli, and PpsR2 from the same bacterium that regulates the same genes in response to light via an interaction with a bacteriophytochrome like antirepressor called BrBphP (Jaubert et al. 2004). *R. capsulatus* contains CrtJ that appears to regulate gene expression by responding directly to the levels of oxygen without any known light control (Masuda et al. 2002). *R. sphaeroides* contains yet a third pattern, in which the repressor PpsR and the blue-light photoreceptor/antirepressor AppA are both involved to regulate gene expression in response to cellular redox and blue-light intensity (Masuda and Bauer 2002). The challenge lies in understanding, at least in part, the observed physiological differences in regulation of gene expression in these different bacteria in terms of the differences in behavior of these protein transcriptional regulators.

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Abbreviations

mV	millivolt
DTNB	5,5'-dithiolbis(2-nitrobenzoic acid)
mBBBr	monobromobimane
DTT	dithiothreitol
GSH	reduced glutathione
GSSG	oxidized glutathione
E_m	midpoint potential
E_h	ambient potential
AMS	4-acetamido-4'-maleimidylstibene-2,2'-disulfonic acid

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Fig. 1.

Amino acid sequence alignments of PpsR/CrtJ proteins. The order of organisms listed from top to bottom and their respective GenBank accession numbers are: *Rhodospirillum rubrum* ZP_00913090, *Rhodospirillum rubrum* S17813, *Jannaschia* sp. CCS1 YP_508106, *Loktanella vestfoldensis* ZP_01002308, *Roseovarius* sp. 217 ZP_01034603, uncultured proteobacterium AAM48684, *Erythrobacter* sp. NAP1 ZP_01041674, gamma proteobacterium KT 71 EAQ96254, *Bradyrhizobium* sp. ORS278 AAT78846, *Bradyrhizobium* sp. ORS278 AAL68701, *Rhodospirillum rubrum* ORF 933 (unpublished), *Rubrivivax gelatinosus* AAO93134, *Rhodospirillum rubrum* palustris YP_570870,

Rhodospirillum rubrum YP_425717, *Thiocapsa roseopersicina* AAX53585, *Thiocapsa roseopersicina* AAX53580. Peptide sequences were aligned with ClustalW, using blosum matrix. All cysteines are highlighted in blue, 100% conserved residues in red, 80% conserved residues in orange, and 60% conserved residues in yellow

Fig. 2.

Oxidation–reduction properties of *R. sphaeroides* PpsR. (A) Oxidation–reduction titration of PpsR at pH 7.0. The open circles represent data obtained from mBBr titrations and the solid line represents the computer best-fit to this data based on the Nernst equation for a two-electron couple. The total DTT concentration was 1.0 mM in 100 mM HEPES buffer at pH 7.0. The redox-equilibration time was 2 h. (B) The dependence of the E_m of PpsR on pH. The solid line is best-fit straight line with a slope of -55 mV/pH, which represents the theoretical slope of -59 mV/pH characteristic of a two-electron process that is accompanied by the uptake of two protons. The vertical error brackets represent the average deviation in the E_m values of measurements for which replicate measurements were carried out. Reaction conditions were

the same as in (A) except with different buffers used over the following pH ranges: MES, pH 5.5–6.5; HEPES, pH 7.0; TRICINE, pH 7.5–8.0; CAPSO, pH 9.0; all at 100 mM concentration

Fig. 3.

Oxidation–reduction properties of *R. sphaeroides* AppA. **(A)** Oxidation–reduction titration of AppA at pH 7.0. The open circles represent data obtained from mBBR titrations under dark condition at 20°C, and the solid line represents the computer best-fit to this data base on the Nernst equation for a two-electron couple. The closed circles represent data under light condition at 5°C and the dashed line represents the computer best-fit to the Nernst equation for a two-electron couple. The total DTT concentration was 2.0 mM in 100 mM HEPES buffer at pH 7.0. The redox-equilibration time was 2 h. **(B)** The dependence of the E_m of AppA on pH. The solid line represents the theoretical slope of -59 mV/pH characteristic of a two-electron process that is accompanied by the uptake of two protons and the dashed line represents

the theoretical slope of -29.5 mV/pH characteristic of a two-electron process that is accompanied by the uptake of one proton. The vertical error brackets represent the average deviation in the E_m values of measurements for which replicate measurements were carried out. Reaction conditions were the same as in (A) except with different buffers used over the following pH ranges: MES, pH 5.5–6.5; HEPES, pH 7.0; TRICINE, pH 7.5–8.0; CAPSO, pH 9.0; all at 100 mM concentration

Table 1

Free thiol group determination of oxidized and reduced PpsR and AppA

Proteins		-SH group
PpsR	Oxidized PpsR	0.0
	Reduced PpsR	2.2 ± 0.3
AppA	Oxidized AppA	5.9 ± 0.4
	Reduced AppA	8.4 ± 0.5