THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 1, Issue of January 5, pp. 601–605, 2001

Printed in U.S.A.

A Single Arginyl Residue in Plastocyanin and in Cytochrome c_6 from the Cyanobacterium *Anabaena* sp. PCC 7119 Is Required for Efficient Reduction of Photosystem I*

Received for publication, August 4, 2000, and in revised form, September 29, 2000
Published, JBC Papers in Press, September 29, 2000, DOI 10.1074/jbc.M007081200

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Positively charged plastocyanin from *Anabaena* sp. PCC 7119 was investigated by site-directed mutagenesis. The reactivity of its mutants toward photosystem I was analyzed by laser flash spectroscopy. Replacement of arginine at position 88, which is adjacent to the copper ligand His-87, by glutamine and, in particular, by glutamate makes plastocyanin reduce its availability for transferring electrons to photosystem I. Such a residue in the copper protein thus appears to be isofunctional with Arg-64 (which is close to the heme group) in cytochrome c_6 from *Anabaena* (Molina-Heredia, F. P., Díaz-Quintana, A., Hervás, M., Navarro, J. A., and De la Rosa, M. A. (1999) *J. Biol. Chem.* 274, 33565–33570) and *Synechocystis* (De la Cerda, B., Díaz-Quintana, A., Navarro, J. A., Hervás, M., and De la Rosa, M. A. (1999) *J. Biol. Chem.* 274, 13292–13297). Other mutations concern specific residues of plastocyanin either at its positively charged east face (D49K, H57A, H57E, K58A, K58E, Y83A, and Y83F) or at its north hydrophobic pole (L12A, K33A, and K33E). Mutations altering the surface electrostatic potential distribution allow the copper protein to modulate its kinetic efficiency: the more positively charged the interaction site, the higher the rate constant. Whereas replacement of Tyr-83 by either alanine or phenylalanine has no effect on the kinetics of photosystem I reduction, Leu-12 and Lys-33 are essential for the reactivity of plastocyanin.

Plastocyanin (Pc),[1] a small single copper protein, and cytochrome c_6 (Cyt), a monoheme protein, function as alternative mobile electron carriers between the two membrane complexes b_6f and photosystem I (PSI) (*cf.* Refs. 1–3 for reviews). The isoelectric point of Pc and Cyt varies widely depending on the organism; the two molecules exhibit the same value of ~9 in the cyanobacterium *Anabaena* sp. PCC 7119 (4).

Their respective three-dimensional structures have repeatedly been solved using proteins from different organisms, and their structure-function relationships have been comparatively analyzed. In Pc, which was investigated first, two active sites were identified: site 1 (or the so-called north hydrophobic pole), located in a flat region around the copper ligand His-87, and site 2 (or the so-called east face), which is referred to as the acidic patch in eukaryotic organisms because it includes aspartic and glutamic residues at positions 42–45 and 59–61 surrounding the solvent-exposed Tyr-83 (1, 2). Recent data indicate that site 2 is responsible for the electrostatic interactions with cytochrome *f* and PSI, whereas site 1, in particular His-87, is involved in electron transfer itself (3, 5).

We have recently reported that Cyt from *Synechocystis* (6) and *Anabaena* (7) possesses two areas equivalent to those of Pc: site 1, which is a hydrophobic region at the edge of the heme pocket providing the contact surface for electron transfer, and site 2, which is a charged patch driving the electrostatic movement toward its two membrane-anchored partners. Our mutagenesis studies of these two cyanobacterial Cyts revealed the existence of a highly conserved arginine residue at position 64, which is located on the protein surface at the frontier between sites 1 and 2, very close to the heme group, that is essential for electron transfer to PSI (6, 7). Arg-64 is the only arginine residue in *Anabaena* Cyt, as is Arg-88 in *Anabaena* Pc. Arg-64 in Cyt could thus be the counterpart of Arg-88 in Pc.

Another peculiarity of Pc and Cyt from *Anabaena*, as compared with the proteins from other sources, is their high isoelectric point (see above) because of their high lysine content. In fact, site 2 is positively charged in both Pc and Cyt, whereas it is typically negative in other cyanobacteria and, in particular, in higher plants (2). Considering that it is site 2 that mainly drives the attractive movement of these two mobile metalloproteins toward the membrane complexes, it was of interest to investigate in *Anabaena* Pc (as previously done in Cyt) how the surface electrostatic potential distribution and redox kinetics are altered by mutations.

This work was thus aimed at comparing the role of specific amino acids in *Anabaena* Pc with those of its counterpart Cyt from the same organism. Pc was modified by mutagenesis of specific residues either at site 1 or site 2, with special attention being paid to Arg-88. The kinetic mechanism of PSI reduction by mutant Pcs was analyzed by laser flash absorption spectroscopy.

EXPERIMENTAL PROCEDURES

Purification of Native Plastocyanin—Pc from *Anabaena* sp. PCC 7119 was purified as described previously (8), with the following two exceptions. i) Pc samples were applied onto the CM-cellulose column after oxidation with potassium ferricyanide, and ii) elution of the adsorbed proteins was performed with a linear gradient of 2–30 mM potassium phosphate buffer, pH 7.0, containing 50 μM potassium ferricyanide. Pc concentration was determined spectrophotometrically using an absorption coefficient of 4.5 mM^{-1} cm^{-1} at 597 nm for the oxidized protein (9).

Construction of Mutants—The mutant *pet*E genes were constructed by the polymerase chain reaction with the QuickChange kit (Strat-

* This research was supported by the Dirección General de Investigación Científica y Técnica (Grant PB96–1381), European Union (Networks ERB-FMRX-CT98–0218 and HPRN-CT1999–00095), and Junta de Andalucía (PAI, CVI-0198). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Pc, plastocyanin; Cyt, cytochrome c_6; PSI, photosystem I; WT, wild-type.

agene) using oligonucleotides of 26–38 bases, 15 ng of DNA templates, and 16 cycles of 12 min in extension time. The construction for the *Anabaena petE* gene previously described was used as a template (4). The DNA fragments were sequenced to check the mutations. Other molecular biology protocols were standard (10).

Production of Recombinant Proteins and Purification Procedures—Transformed cells of *Escherichia coli* MC1061 were grown in a 40-liter fermentor (Biostat C, B. Braun Biotech) at 37 °C for 18 h under aerobic conditions. The fermentor was filled with 20 liters of standard Luria-Bertani medium (10) supplemented with 100 μg/ml ampicillin and 200 μM $CuSO_4$. The air flow was adjusted automatically to keep the concentration of dissolved oxygen at ~95% of its saturation value. The culture was stirred at 250 rpm. The pH value was kept constant at ~6.0 by addition of small amounts of 1 N HCl. To prevent loss of the expression vector, *E. coli* cells were transformed and immediately used to inoculate the reactor.

Cells were collected by tangential filtration in a Sartocon cross-flow filtration system (Sartorius), and the periplasmic fraction was extracted by freezing the cell paste at −20 °C. The frozen cell paste was resuspended in 100 ml of deionized water and centrifuged. The resulting suspension was extensively dialyzed against 2 mM potassium phosphate, pH 7.0, with the exception of mutants K33E, H57E, K58E, and R88E, which were dialyzed against 2 mM Tris-HCl, pH 8.0. From this point on, the purification procedure for most of the mutants was identical to that for native Pc, with minor changes in elution gradients. The mutants K33E, H57E, and K58E were applied onto a DEAE-cellulose column equilibrated with 2 mM Tris-HCl, pH 8.0. Elution of the adsorbed proteins was performed with a linear gradient of 0–100 mM NaCl in the same buffer. R88E was purified by gel filtration in a Sephadex G-50 column. In all cases, 50 μM potassium ferricyanide was added to the gradient buffers to keep Pc oxidized. Protein concentration was determined as described above.

Redox Titrations—The redox potential value for each Pc mutant was determined as reported previously (4, 11) by following the differential absorbance changes at 597 minus 500 nm. Errors in the experimental determinations were less than 10 mV.

Preparation of PSI Particles—PSI particles were isolated from *Anabaena* cells by β-dodecyl maltoside solubilization (12, 13). The chlorophyll:P700 ratio of the resulting PSI preparations was ~140:1. The P700 content in PSI samples was calculated from the photoinduced absorbance changes at 820 nm using the absorption coefficient of 6.5 mM^{-1} cm^{-1} determined by Mathis and Sétif (14). Chlorophyll concentration was determined according to the method of Arnon (15).

Laser-flash Absorption Spectroscopy—Kinetics of flash-induced absorbance changes in PSI were followed at 820 nm as described previously (16). The standard reaction mixture and other experimental conditions have been reported (17). The buffer used throughout this work was 20 mM Tricine/KOH, pH 7.5. Data collection, as well as kinetic and thermodynamic analyses, were carried out as reported previously (16, 18). Apparent thermodynamic parameters were estimated as in Díaz *et al.* (19) by fitting the experimental data to the equation of Watkins *et al.* (20). Experimental errors were less than 10% for both the kinetic constants and thermodynamic parameters.

Structure Simulations—Structure and surface electrostatic potential distribution of Pc mutants were modeled using the Swiss-Pdb Viewer program (21). The solution structure of reduced Pc from *Anabaena variabilis* (22), whose amino acid sequence exhibits 100% identity with that of Pc from *Anabaena* sp. PCC 7119, was used as a template. The quality of the modeled structures for each Pc mutant was tested using the PROCHECK program (23).

RESULTS AND DISCUSSION

Seven residues of *Anabaena* Pc were chosen to be mutated, two at the north hydrophobic patch and five at the east charged area (Fig. 1). The steric hindrance and nature of interactions at site 1 were investigated by replacing Leu-12 by Ala, and Lys-33 by Ala or Glu. The functional equivalence of site 2 in *Anabaena* Pc and in the negatively charged copper proteins was analyzed by substituting residues at positions 49, 57, and 58; Asp-49 was replaced by Lys, and both His-57 and Lys-58 were replaced by either Ala or Glu. Tyr-83 was changed to Ala and Phe to check whether Tyr-83 is involved in redox reactions, a role that was first proposed by He *et al.* (24) but later discarded by Bendall *et al.* (25) in other Pcs.

Our analysis of all primary sequences of Pcs deposited at the Protein Data Bank with the BLAST search program (26) revealed that Arg-88 is conserved in cyanobacteria but not in higher plants or in green algae. The reason for such conservation of Arg-88 in cyanobacterial but not in eukaryotic Pcs was thus investigated by replacing Arg-88 with either glutamine, as it is in spinach, or with glutamate.

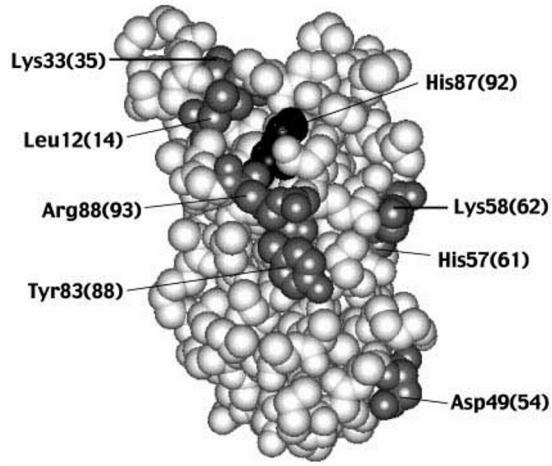

FIG. 1. **Space-filling model of *Anabaena* plastocyanin showing the residues modified by mutagenesis.** The molecule is oriented with its typical east face (electrostatic area around Tyr-83; site 2) in *front*, whereas the north hydrophobic patch (or site 1) is at the *top*. The mutant residues are depicted in *gray*, and His-87 is depicted in *black*. Each residue is identified with two numbers, the first corresponding to its position in the copper protein from higher plants and the second (between parentheses) corresponding to its place in the amino acid sequence of *Anabaena* plastocyanin.

Neither the electronic absorption spectrum nor the midpoint redox potential of *Anabaena* Pc was altered by the mutations mentioned above, thereby revealing that the copper center was not distorted. As shown in Table I, the only exceptions were the R88E and K33E mutants, whose midpoint redox potential values are 25–30 mV lower than that of WT Pc.

The kinetic profile of PSI reduction under standard conditions was monoexponential with all mutants, as it is with WT Pc (16), but the rate constants varied greatly. The observed pseudo first-order rate constant (k_{obs}) of PSI reduction by any mutant is linearly dependent on Pc concentration, as is the case with the WT molecule (Fig. 2). This finding can be interpreted by assuming that there is no formation of a detectable transient complex between PSI and Pc, in agreement with a collisional kinetic model (3, 16).

The bimolecular rate constant for the overall reaction (k_{bim}) can be calculated under standard conditions from the linear plots of k_{obs} *versus* Pc concentration. Table I shows that most mutants yield k_{bim} values smaller than WT Pc, with the exception of D49K, which exhibits a higher reactivity, and the two mutants at Tyr-83, which are slightly affected. As expected, all mutants of the hydrophobic patch suffer a significant decrease in their k_{bim} value. Electrostatic mutations at positions 33, 57, and 58 cause *Anabaena* Pc to decrease its availability to reduce PSI as much as its global charge is made more negative. Major changes are obtained with the Arg-88 mutants, because replacement of Arg-88 with glutamine or glutamate induces a decrease in k_{bim} of ~8 and 24 times, respectively (Table I). Fig. 2 shows how inefficient the R88Q and R88E mutants are, even at high concentrations, compared with the WT molecule.

Taking into account the electrostatic nature of the interactions between Pc and PSI, a detailed analysis of the effect of ionic strength on k_{bim} was performed. The k_{bim} values of WT Pc and all its mutants decrease steadily with increasing NaCl concentration, indicating the existence of attractive electro-

TABLE I

Midpoint redox potential (E_m) of wild-type and mutant plastocyanins, as well as bimolecular rate constants and activation parameters for the overall reaction of PSI reduction by the different copper proteins

Plastocyanin	E_m, pH 7.0	$k_{bim} \times 10^{-7}$	$k_{inf} \times 10^{-6}$	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
	mV	$M^{-1}\ s^{-1}$	$M^{-1}\ s^{-1}$	*kJ mol*$^{-1}$	*kJ mol*$^{-1}$	*J mol*$^{-1}$ *K*$^{-1}$
Wild-type	355	7.68	5.96	27.9	36.2	27.7
Mutants at the east face						
R88E	325	0.32	ND[a]	35.9	36.5	2.1
R88Q	336	0.99	5.78	33.0	41.4	33.0
D49K	353	9.73	6.29	27.5	35.3	26.2
H57A	345	1.70	6.09	31.7	39.1	24.7
H57E	341	3.76	5.94	29.6	39.3	32.5
K58A	346	3.03	3.48	30.3	41.1	36.3
K58E	339	1.59	4.19	31.7	42.9	37.4
Y83A	340	4.44	6.18	29.3	44.0	49.2
Y83F	343	4.73	5.22	29.1	34.0	16.5
Mutants at the north pole						
L12A	371	1.37	0.84	32.2	31.0	−4.2
K33A	355	2.55	3.75	30.7	40.2	31.9
K33E	330	1.35	3.10	32.2	41.9	32.3

[a] ND, not determined, because this mutant shows a k_{bim} independent of ionic strength.

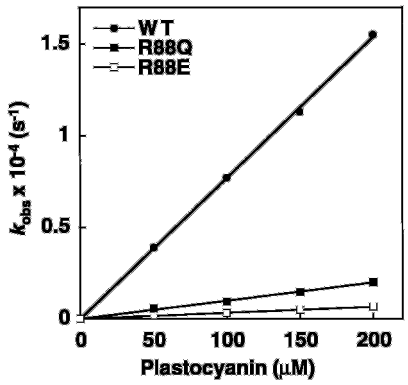

FIG. 2. **Dependence upon plastocyanin concentration of the observed rate constant (k_{obs}) for PSI reduction by the WT species and R88 mutants of the copper protein.** Experimental conditions were as described under "Experimental Procedures."

static interactions between the reaction partners that are overwhelmed at high ionic strength. The only exception is the R88E mutant, for which the k_{bim} values are independent of ionic strength (see Fig. 4).

The formalism developed by Watkins *et al.* (20) facilitates the analysis of the intrinsic reactivity of redox partners in the absence of electrostatic interactions. By applying the Watkins equation to our data on the ionic strength dependence of k_{bim}, values for the bimolecular rate constant extrapolated to infinite ionic strength (k_{inf}) can be calculated. As shown in Table I, all mutants, except L12A and R88E, exhibit k_{inf} values similar to that of WT Pc, indicating that the minor reactivity of Pc mutants under standard conditions is mainly due to electrostatic rather than hydrophobic or structural changes. In the case of L12A, however, the k_{inf} value is 7-fold lower than that for WT Pc. This suggests that such an isoelectric mutation may indeed induce structural changes hindering the interaction between Pc and PSI, as previously observed in *Synechocystis* (17) and spinach (27). The Watkins equation, however, cannot be applied as such to R88E because its interactions with PSI are independent of ionic strength. The k_{bim} value for R88E at any NaCl concentration (1.46×10^6 M^{-1} s^{-1}) is 4-fold lower than the k_{inf} value for WT Pc (see Table I). This suggests that the electrostatic change induced by mutation is not the only factor affecting the reactivity of R88E toward PSI. Worth mentioning is the finding that the R64E mutant of *Anabaena* Cyt exhibits a k_{inf} value that is also 4- or 5-fold lower than that for WT Cyt (7).

The nature of interactions between PSI and Pc was further investigated by performing a thermodynamic analysis of PSI reduction by the copper protein mutants. In all cases, the temperature dependence of the observed rate constant yielded linear Eyring plots with no breakpoints, from which the values for the apparent activation enthalpy ($\Delta H^{\ddagger}$), entropy ($\Delta S^{\ddagger}$), and free energy ($\Delta G^{\ddagger}$) of the overall reaction can be calculated. As shown in Table I, the most significant difference is observed with the R88E mutant, whose free energy change is 8.0 kJ mol^{-1} higher than that of WT Pc, as expected from its impaired reactivity toward PSI. Such a difference in $\Delta G^{\ddagger}$ is mainly due to a decrease of 25.6 J mol^{-1} K^{-1} in the entropic term. Also interesting is the L12A mutant, which exhibits a free energy change of 4.3 kJ mol^{-1} higher than that of WT Pc that is mainly due to the decrease in the entropic term. The observed changes in $\Delta G^{\ddagger}$ with the other mutants can be attributed to shifts in the enthalpic and/or entropic terms.

To elucidate whether the lower reactivity of R88E can be ascribed to reasons other than the electrostatic change itself, its availability to transfer electrons to PSI from spinach and *Synechocystis* was analyzed in comparison with *Anabaena* WT Pc. The k_{bim} value for spinach PSI reduction was 3.5×10^5 M^{-1} s^{-1} with R88E, or 4.3-fold lower than that with *Anabaena* WT Pc (1.5×10^6 M^{-1} s^{-1}). The k_{bim} value for *Synechocystis* PSI reduction was 1.9×10^6 M^{-1} s^{-1} with R88E, or 15-fold lower than that with *Anabaena* WT Pc (2.8×10^7 M^{-1} s^{-1}). These findings allow us to conclude that Arg-88 is a crucial residue not only for long range electrostatic attractions between Pc and PSI but also for electron transfer. It should be noted that glutamine at position 88 of spinach Pc has been replaced with asparagine, glutamate, lysine, and tyrosine with no significant changes in the kinetics of PSI reduction (27, 28).

Divalent cations like Mg^{2+} have previously been reported to be specifically involved in Pc/PSI interactions in other organisms, but not *Anabaena* (8). As such, an analysis of the effect of Mg^{2+} cations on the bimolecular rate constant of PSI reduction by WT and mutant Pcs was performed. The experimental results showed that the three mutants with one positive charge replaced by a negative residue (K33E, K58E, and R88E) are the only ones altered by magnesium cations. With these mutants the bimolecular rate constant exhibits higher values with $MgCl_2$ than with NaCl at the same ionic strength (data not shown). All these findings can be interpreted by assuming not only that the added negative charges in Pc mutants facilitate the formation of new salt bridges with positive counterparts in PSI (16), but also that the original positive residues in WT Pc interact with an acidic area in PSI.

The positively charged site 2 in *Anabaena* Pc does appear to

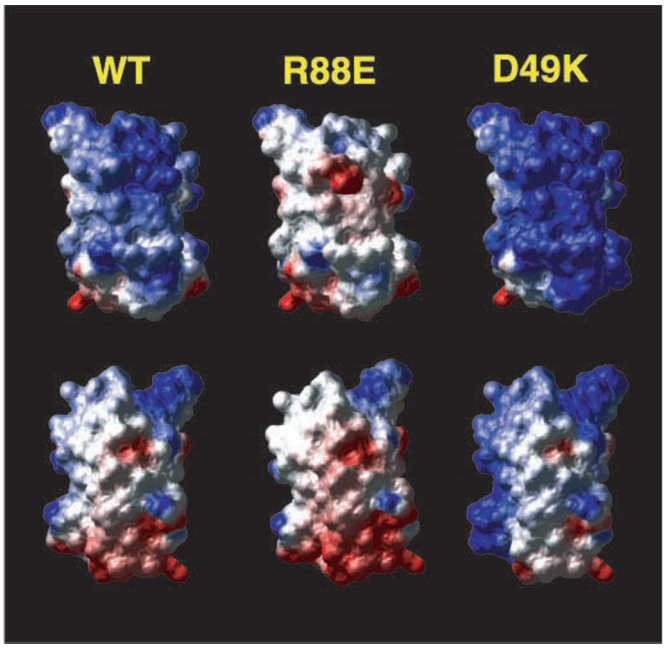

FIG. 3. **Surface electrostatic potential distribution of wild-type plastocyanin (*WT*) and its mutants R88E and D49K.** The electrostatic potential was calculated as indicated under "Experimental Procedures." Simulations were performed assuming an ionic strength of 40 mM at pH 7.0. Negative and positive potential regions are depicted in *red* and *blue*, respectively. Protein molecules are in the same orientation as in Fig. 1 (*upper*) or rotated 180° around the vertical axis (*lower*).

play the same role as the negatively charged site in other Pcs. The surface electrostatic potential distribution of *Anabaena* Pc was then calculated for its WT species as well as for the D49K and R88E molecules. As can be seen in Fig. 3, their respective electrostatic charges at site 2 correlate well with their relative efficiency as electron donors to PSI; the more positive the local surface charge, the higher the kinetic rate constant of photosystem reduction. A similar correlation had previously been observed with a number of mutants at site 2 of Cyt from *Anabaena* (7) and *Synechocystis* (6).

The close similarity between Pc and Cyt from *Anabaena* was investigated by comparing the structures of the two molecules and the relative position of critical surface residues. The three-dimensional structures of the metalloproteins were thus modelled (Fig. 4, *upper panel*). Assuming that His-87 in Pc plays the same redox role as the heme group in Cyt (29), the comparison of the two protein structures makes Arg-88 and Asp-49 of Pc occupy positions equivalent to Arg-64 and Asp-72 in Cyt. In addition, the high number of lysine residues in both molecules could contribute to site 2 being so positively charged.

Such a structural coincidence between Pc and Cyt from *Anabaena* is indeed supported by the experimental findings concerning the kinetic behavior of mutants affected at the arginine and aspartate residues. Fig. 4 (*lower panels*) shows how the k_{bim} values at low ionic strength for the D49K mutant of Pc are significantly higher than those for WT Pc, as are the values for the D72K mutant of Cyt compared with WT Cyt. The k_{bim} values for R88E of Pc and R64E of Cyt are, however, lower than those for their respective WT species. Also worth noting is the parallel ionic strength dependence of k_{bim} with the WT and mutant proteins, *i.e.* the decrease in k_{bim} with increasing NaCl concentration.

Several possibilities concerning the role of the single arginyl residue in both Cyt and Pc can be devised. The interaction of these two metalloproteins with PSI should involve the formation of a transient electrostatic complex, which has in fact kinetically been detected with Cyt but not with Pc, probably

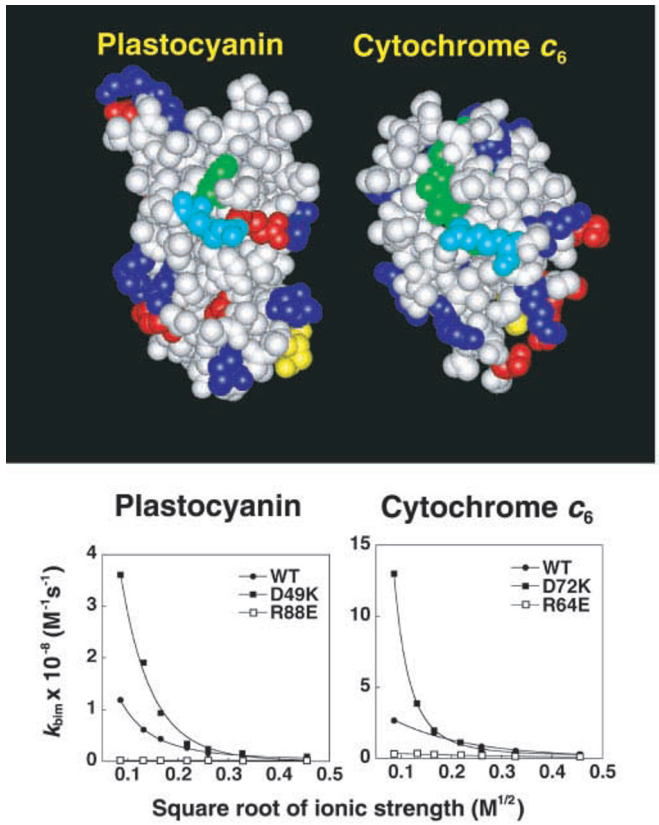

FIG. 4. *Upper panel*, space-filling model of plastocyanin and cytochrome c_6 from *Anabaena* with the acidic and basic residues in *red* and *blue*, respectively. His-87 in Pc and the heme group in Cyt are depicted in *green*, Arg-88 in Pc and Arg-64 in Cyt are in *light blue*, and Asp-49 in Pc and Asp-72 in Cyt are in *yellow*. The two molecules are oriented with their respective "east" faces (site 2) just in *front* and the so-called north hydrophobic poles (site 1) at the *top*. *Lower panels,* effect of ionic strength on k_{bim} for PSI reduction by WT Pc and its mutants D54K and R88E (*left*) as well as by WT Cyt and its mutants D49K and R64E (*right*). Experimental conditions for the Pc species were as described under "Experimental Procedures," with the ionic strength being adjusted to the desired value by adding small amounts of a concentrated NaCl solution. The data for Cyt are from Molina-Heredia *et al*. (7).

due to its low association constant. The arginyl residue could thus be required for appropriate orientation of the redox centers within the transient complex. Specific electrostatic interactions of Arg-88 in Pc and Arg-64 in Cyt with negatively charged groups in PSI could be made evident as soon as a high resolution structure for PSI is made available. In addition, the proximity of the arginyl residue to the prosthetic group in each protein can explain why the arginine mutants of both Pc and Cyt exhibit values for the rate constant extrapolated to infinite ionic strength lower than those of the respective WT species. In this context, conformational changes of Arg-88 related to changes in the redox state of Pc have been described recently (30).

To conclude, we can say that site 1 of *Anabaena* Pc is similar to site 1 of other Pcs, but site 2 is positively charged in *Anabaena*, whereas it is negatively charged in other organisms. In addition, not only Pc but also Cyt from *Anabaena* contains a single arginine residue between sites 1 and 2 that appears to play the same function in the two molecules.

Acknowledgments—We thank S. Alagaratnam, A. Balme, A. Díaz, and S. Murdoch for critically reading the manuscript.

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