Reaction of Cytochrome c_2 with Photosynthetic Reaction Centers from *Rhodopseudomonas viridis*†

David B. Knaff,*,‡ Anne Willie,§ Joan E. Long,§,∥ Aidas Kriauciunas,‡,⊥ Bill Durham,*,§ and Francis Millett*,§

Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061, and Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701

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ABSTRACT: The reactions of *Rhodopseudomonas viridis* cytochrome c_2 and horse cytochrome *c* with *Rps. viridis* photosynthetic reaction centers were studied by using both single- and double-flash excitation. Single-flash excitation of the reaction centers resulted in rapid photooxidation of cytochrome *c*-556 in the cytochrome subunit of the reaction center. The photooxidized cytochrome *c*-556 was subsequently reduced by electron transfer from ferrocytochrome c_2 present in the solution. The rate constant for this reaction had a hyperbolic dependence on the concentration of cytochrome c_2, consistent with the formation of a complex between cytochrome c_2 and the reaction center. The dissociation constant of the complex was estimated to be 30 μM, and the rate of electron transfer within the 1:1 complex was 270 s^{-1}. Double-flash experiments revealed that ferricytochrome c_2 dissociated from the reaction center with a rate constant of greater than 100 s^{-1} and allowed another molecule of ferrocytochrome c_2 to react. When both cytochrome *c*-556 and cytochrome *c*-559 were photooxidized with a double flash, the rate constant for reduction of both components was the same as that observed for cytochrome *c*-556 alone. The observed rate constant decreased by a factor of 14 as the ionic strength was increased from 5 mM to 1 M, indicating that electrostatic interactions contributed to binding. Molecular modeling studies revealed a possible cytochrome c_2 binding site on the cytochrome subunit of the reaction center involving the negatively charged residues Glu-93, Glu-85, Glu-79, and Glu-67 which surround the heme crevice of cytochrome *c*-554. The photooxidation of horse cytochrome *c* by *Rps. viridis* reaction centers was much slower than that of *Rps. viridis* cytochrome c_2, and obeyed second-order kinetics with a rate constant of 1.7×10^5 M^{-1} s^{-1}. The reaction of cytochrome c_2 with reaction centers in membranes prepared from *Rps. viridis* cells was also studied, with results similar to those reported above using purified reaction centers.

The initial light-driven step in photosynthetic purple bacteria involves electron transfer from a bacteriochlorophyll dimer to a bacteriopheophytin within the reaction center complex (Kirmaier & Holten, 1987; Parson, 1987). Electron transfer proceeds from reduced bacteriopheophytin to a primary quinone, Q_A, which can accept only one electron, and then from Q_A^- to a secondary quinone, Q_B (Crofts & Wraight, 1983). A soluble cytochrome c_2 serves as the immediate electron donor to the photooxidized bacteriochlorophyll dimer in the three-subunit reaction centers found in *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and *Rhodospirillum rubrum* (Dutton & Prince, 1978; Crofts & Wraight, 1983; Amesz & Knaff, 1988; Rickle & Cusnaovich, 1979). The multiphasic kinetics observed for cytochrome c_2 oxidation by *Rb. sphaeroides* reaction centers have been attributed to the reactions of two different bound forms of cytochrome c_2 in addition to cytochrome c_2 in solution (Overfield et al., 1979; Rosen et al., 1979; Overfield & Wraight, 1986; Moser & Dutton, 1988). Moser and Dutton (1988) found that oxidized horse cytochrome *c* inhibited the reaction of reduced cytochrome *c*, suggesting the formation of a product complex. Tiede (1987) used transient linear dichroism techniques to detect a difference in the binding orientations of horse cytochrome *c* and cytochrome c_2 on *Rb. sphaeroides* reaction centers which might account for the difference in reaction rates.

In contrast to the systems discussed above, one of the hemes in a four-heme, reaction center associated cytochrome is the direct reductant for the bacteriochlorophyll dimer in *Rhodopseudomonas viridis* (Case et al, 1970; Dracheva et al., 1986; Shopes et al., 1987), *Rhodocyclus gelatinosus* (Dutton & Prince, 1978; Matsuura et al, 1988; Fukushima et al., 1988), and two species of *Chromatium* (Parson, 1969; Parson & Case, 1970; Dutton & Prince, 1978; Crofts & Wraight, 1983; Garcia et al., 1987). The determination of the X-ray crystal structure of the reaction center from *Rps. viridis* has provided detailed information on the structural organization of the prosthetic groups within the protein (Deisenhofer et al., 1985). The four hemes are arranged in an approximately linear fashion in the cytochrome subunit, which protrudes into the periplasmic space of the bacterium (Figure 1). Recent EPR and kinetic studies have indicated that the hemes are arranged in the order BChl dimer, *c*-559, *c*-552, *c*-556, *c*-554 (Nitschke & Rutherford, 1989; Dracheva et al., 1988; Vermeglio et al., 1989). Redox titration studies have shown that the room temperature midpoint potentials of the four hemes are the following: *c*-559, +360 mV; *c*-552, +20 mV; *c*-556, +312 mV; *c*-554, <−50 mV (Shinkarev et al., 1990; Dracheva et al., 1986; Alegria & Dutton, 1987). It is interesting that the low-potential heme *c*-552 is positioned between the two high-potential hemes, since only the latter are reduced under physiological conditions. Kinetic studies have shown that the highest potential heme,

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* To whom correspondence should be addressed.
‡ Texas Tech University.
§ University of Arkansas.
∥ Present address: Eastman Kodak, Batesville, AR 72501.
⊥ Present address: Eli Lilly Laboratories, Indianapolis, IN 46285.

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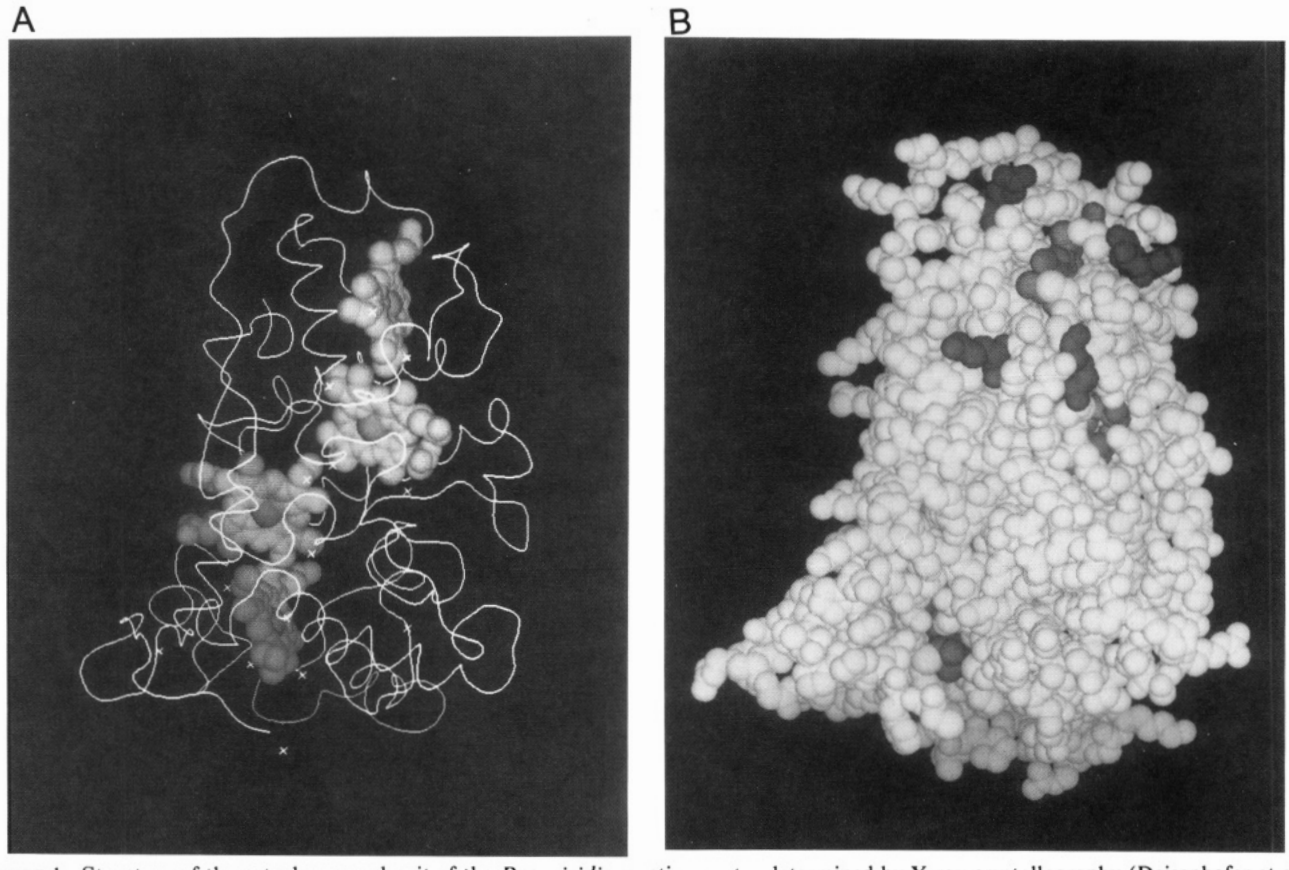

FIGURE 1: Structure of the cytochrome subunit of the *Rps. viridis* reaction center determined by X-ray crystallography (Deisenhofer et al., 1985; protein data bank 1PRC). The molecule has been rotated approximately 180° about the vertical axis relative to Figures 1b and 5 of Deisenhofer et al. (1985), and the other subunits (not shown) are located below the cytochrome subunit. The heme groups are *c*-559, *c*-552, *c*-556, and *c*-554, from bottom to top. (A) The polypeptide backbone of the cytochrome subunit is represented by a thread, while the heme groups are represented by CPK space-filling models. The positions of the sulfur atoms in the thioether bridges and free cysteines are indicated by x's. (B) Space-filling model, with all residues colored white, except the heme groups and the carboxylate residues surrounding the cytochrome *c*-554 heme group, which are colored grey. These are Asp-92 and Glu-93 to the immediate right of the *c*-554 heme group, Glu-85 to the bottom right, Glu-79 to the bottom left, and Glu-67 to the top left. The models were obtained on a Silicon Graphics W-4D25G workstation using BIOSYM Insight software.

c-559, is oxidized the most rapidly by the photooxidized bacteriochlorophyll dimer, with a half-time of about 0.19 μs (Shopes et al., 1987; Dracheva et al., 1988). Subsequent electron transfer from *c*-556 to *c*-559 occurs with a half-time of 2.5 μs.

Shill and Wood (1984) have presented evidence that cytochrome c_2 is an electron donor for the high-potential reaction center associated cytochromes in *Rps. viridis*. A soluble cytochrome has also been implicated as the electron donor for the reaction center associated high-potential cytochrome *c* in *Chromatium vinosum* (van Grondelle et al., 1977) and *Rhodocylus gelatinosus* (Matsuura et al., 1988). However, relatively little information is available on the interaction of soluble *c*-type cytochromes with four-subunit, cytochrome *c* containing reaction centers. We report below the results of kinetic studies on the reaction of *Rps. viridis* reaction centers with *Rps. viridis* cytochrome c_2 and horse cytochrome *c*.

Experimental Procedures

Reaction centers were isolated from *Rps. viridis*, according to a modification of the procedure of Prince et al. (1977). Some reaction center samples were generous gifts from Drs. D. Holten, C. Kirmaier, G. Alegria, and D. Oesterhelt. The reaction centers had absorbance spectra and peptide compositions essentially identical with those reported in the literature (Prince et al., 1977; Thornber et al., 1980; Clayton & Clayton, 1978). Membrane fragments were isolated from *Rps. viridis* cells by sonication as described by Carithers and Parson (1975) and Pucheu et al. (1973). The cells were washed once in 30 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 8, sonicated at 0 °C for five 1-min periods with a Heat Systems W185 sonicator, and centrifuged at 10000*g* for 15 min to remove cell debris. The supernatant was centrifuged at 130000*g* for 3 h to collect the membrane fragments. Cytochrome c_2 was purified from *Rps. viridis* by a modification of the method of R. G. Bartsch (1978), in which the concentration on an Amicon YM-5 ultrafiltration membrane replaced the ammonium sulfate precipitate step. Sodium dodecyl sulfate gel electrophoresis of cytochrome c_2 on a 10–15% gel followed by silver staining resulted in a single band with an apparent molecular weight of 12.5K. A single band with p*I* = 8.1 was observed after isoelectric focusing. Gel filtration of cytochrome c_2 on Sephadex G-75 gave a single symmetrical elution peak. All fractions in this peak had identical A_{280}/A_{416} ratios of 0.17 for the ascorbate-reduced protein. The apparent molecular weight of this peak was determined to be 12.5K (Andrews, 1965), indicating that cytochrome c_2 was monomeric at 200 mM ionic strength. The α-band 550.2-nm extinction coefficients of oxidized and reduced *Rps. viridis* cytochrome c_2 were calculated to be 9.2 and 28.8 mM^{-1} cm^{-1}, respectively, from a stock solution of known absorbance after determination of the heme *c* concentration

[1] Abbreviations: TFA, trifluoroacetyl; TFC, (trifluoromethyl)-phenylcarbamoyl; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; LDAO, lauryldimethylamine oxide detergent; Tris, tris(hydroxymethyl)aminomethane.

using the alkaline pyridine hemochromogen method (Takaichi & Morita, 1981). Horse cytochrome *c* (type VI) was purchased from Sigma Chemical Co. Lauryl-*N*,*N*-dimethylamine *N*-oxide (LDAO) was a gift from Onyx Chemical Co. (Jersey City, NJ).

Bacteriochlorophyll *b* was determined after extraction into 7:2 (v/v) acetone/methanol (Jones & Saunders, 1972). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Laemmli (1977), and the gels were stained for protein with Coomassie Brilliant Blue. Absorbance spectra were measured with an Aminco DW-2a spectrophotometer.

Kinetics of the reaction of ferrocytochrome c_2 with *Rps. viridis* reaction centers after single or double flashs were measured by using a flash photolysis system consisting of two xenon flash lamps placed 2 cm on each side of a semimicro glass fluorescence cuvette. The pulse width of each flash lamp was 25 μs, and the delay between the two flashes could be varied continuously. In some experiments, a Phase-R laser operating at a wavelength of 590 nm and a pulse width of 0.5 μs was substituted for the first xenon flash lamp. Both the laser beam and the xenon flash lamp beam illuminated the entire volume of the sample cuvette used for the probe beam, and the intensities of both flashes were adjusted to saturate the photooxidation signal. The probe beam came from a 100-W tungsten halogen lamp and was passed through an interference filter, collimated, and focused on the sample with a fused silica lens. The probe beam then passed through a monochromator (Kratos GM252) and was detected with an R928 photomultiplier tube. The signal was recorded on a 1010 Biomation Waveform recorder and transferred to an IBM PC for kinetic analysis using a linear least-squares program. The reaction mixtures contained 1 μM reaction centers, 1–50 μM ferrocytochrome c_2 in 5 mM Tris-HCl, pH 8.0, 0.025% LDAO, 100 μM 2,3-dimethyl-5-methyl-1,4-benzoquinone (Q_0, obtained from Sigma Chemical Co.), and 1 mM sodium ascorbate. In some experiments, 20 μM ubiquinone Q_{10} (obtained from Sigma Chemical Co.) was used in place of Q_0.

Results

Photooxidation of Rps. viridis Cytochrome c_2 by Rps. viridis Reaction Centers. Excitation of *Rps. viridis* reaction centers with a single saturating flash in the absence of cytochrome c_2 resulted in rapid oxidation of the cytochromes in the heme subunit, as described by Dracheva et al. (1988) and Shopes et al. (1987). The 556-nm absorbance transient shown in Figure 2A was obtained when both cytochromes *c*-556 and *c*-559 were initially maintained in the reduced state by 1 mM ascorbate, and the short-chain ubiquinone Q_0 was present in the buffer. The absorbance at 556 nm was bleached within the response time of the instrument (25 μs) and then slowly returned to the preflash level with a rate constant of 1.1 s^{-1}. The wavelength dependence of the rapid absorbance change between 0 and 25 μs, shown in Figure 2B, indicated that it was due to the oxidation of cytochrome *c*-556, as shown by Dracheva et al. (1988) and Shopes et al. (1987). If the reaction centers were subjected to a second saturating flash 110 ms after the first saturating flash, an additional rapid decrease in absorbance was observed (Figure 2A). The wavelength dependence of this additional decrease (Figure 2B) indicated that it was largely due to the photooxidation of cytochrome *c*-559. This additional decrease in absorption was small if the delay between the first and second flashes was less than 0.5 ms and increased to its maximum value as the delay was increased to 40 ms (Figure 2C). Presumably, this dependence on the delay time reflects the electron-transfer reaction from

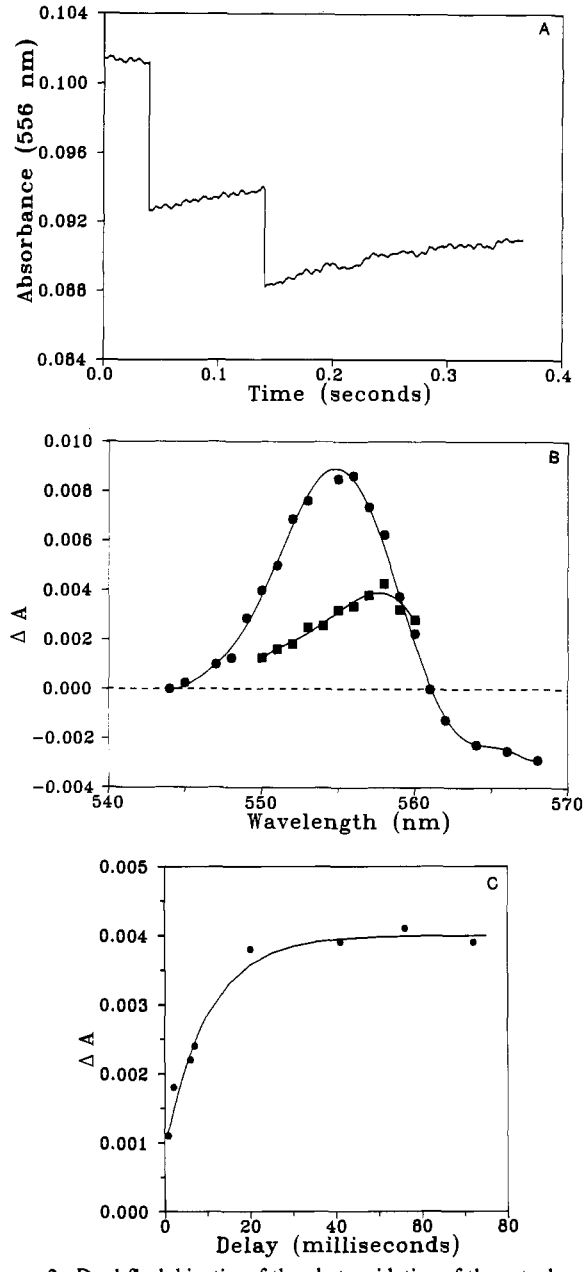

FIGURE 2: Dual-flash kinetics of the photooxidation of the cytochromes in the heme subunit of *Rps. viridis* reaction centers. A solution containing 1 μM reaction centers in 5 mM Tris-HCl, pH 8.0, 0.025% LDAO, 1 mM sodium ascorbate, and 100 μM Q_0 was excited with two saturating 25-μs xenon flashes separated by 110 ms. (A) Transient absorbance signal detected at 556 nm. (B) Wavelength dependence of the photooxidation of the cytochromes in the heme subunit. (●) The absorbance change immediately before and after the first flash. (■) The absorbance change from immediately after the first flash to immediately after the second flash. The delay time was 73 ms. (C) Dependence of the absorbance change on the second flash as a function of the delay time between the two flashes.

the endogenous menaquinone at the Q_A site to Q_0 at the Q_B site. The amplitude of this additional cytochrome *c*-559 oxidation was much smaller in the absence of Q_0 (data not shown), indicating that the natural ubiquinone present at the Q_B site was largely lost during purification (Gao et al., 1990). Experiments were also carried out using the long-chain ubiquinone Q_{10} in place of Q_0, and replacing the first xenon flash with a laser flash with a lifetime of 0.5 μs. When both flashes were saturating, the relative amplitudes and wavelength dependencies of the two transients were essentially the same as in the experiments using Q_0 described in Figure 2A,B. However, the amplitude of the absorbance change following

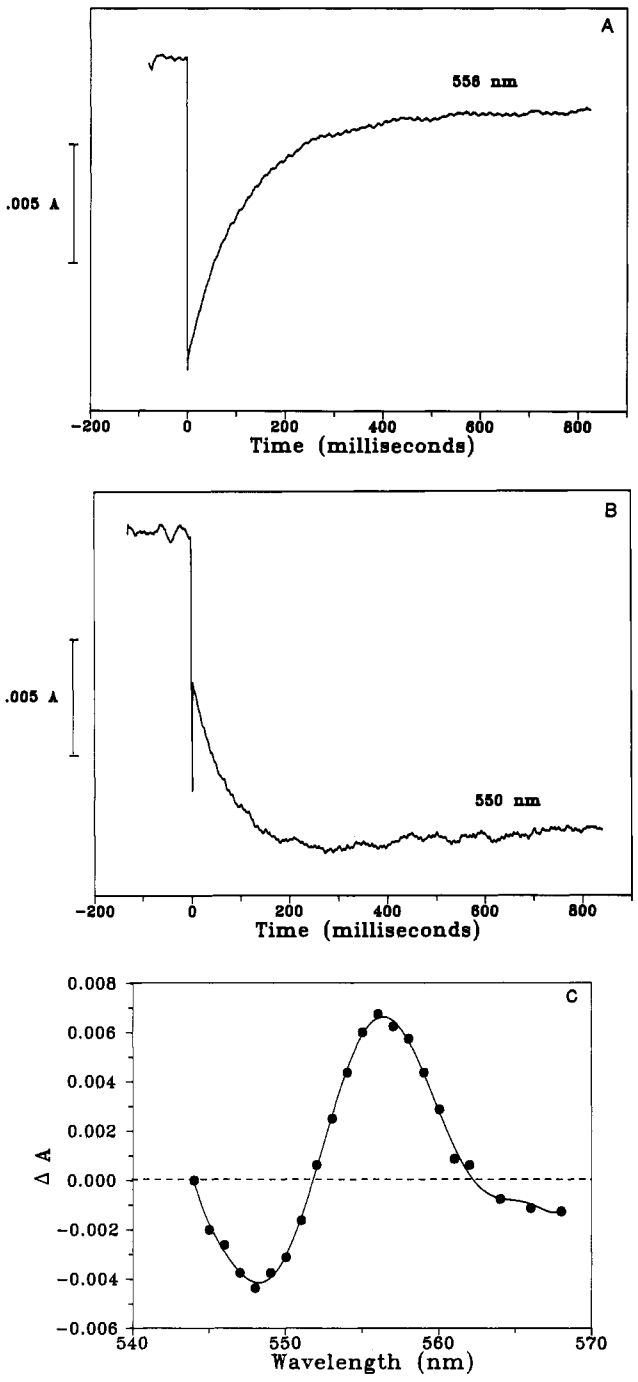

FIGURE 3: Single-flash kinetics of the reaction between cytochrome c_2 and cytochrome c-556 in the reaction center. The reaction mixture contained 0.5 μM reaction centers and 1 μM cytochrome c_2 in 20 mM Tris-HCl, pH 8.0, 0.025% LDAO, 1 mM sodium ascorbate, and 40 μM Q_0. (A) Absorbance transient at 556 nm. (B) Absorbance transient at 550 nm. (C) Wavelength dependence of the absorbance change from 25 μs to 400 ms after the flash.

the second flash was independent of the delay between the two flashes down to 1-ms delay.

In the presence of 1 μM reduced cytochrome c_2, the 556- and 550-nm absorbance transients each consisted of a fast phase between 0 and 25 μs followed by a slow phase between 25 μs and 400 ms with a rate constant of 10 s^{-1} (Figure 3A,B). The fast phase at each wavelength was identical with that in the absence of cytochrome c_2 (Figure 2B), and was due to the photooxidation of cytochrome c-556 (Shopes et al., 1987). The wavelength dependence of the slow phase had a maximum at 556 nm and a minimum at 548 nm (Figure 3C), consistent with electron transfer from cytochrome c_2 to cytochrome c-556.

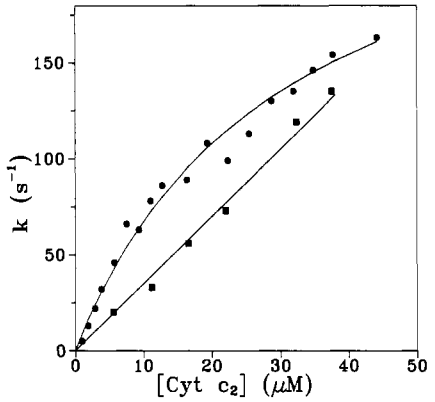

FIGURE 4: Concentration dependence of single-flash kinetics of the reaction between cytochrome c_2 and purified reaction centers (●) or *Rps. viridis* membranes (■). For the purified reaction center experiments, the reaction mixture contained 1 μM reaction centers, 1–44 μM cytochrome c_2 in 10 mM Tris-HCl, pH 8.0, 0.025% LDAO, 1 mM sodium ascorbate, and 100 μM Q_0. The observed first-order rate constant of the absorbance transient at 556 nm is plotted vs the cytochrome c_2 concentration. The solid line is the best fit to eq 1 with k_{et} = 270 s^{-1} and K_d = 30 μM. For the membrane experiments, the reaction mixture contained membranes with 1 μM reaction centers, 5–38 μM cytochrome c_2 in 20 mM Tris-HCl, pH 8.0, and 1 mM sodium ascorbate.

The rate constant for this reaction was found to have a hyperbolic dependence on the concentration of cytochrome c_2, suggesting the formation of a complex between cytochrome c_2 and the reaction center (Figure 4). The observed rate constants were fitted to the equation:

$$k_{obsd} = k_{et}[c_2]/(K_d + [c_2]) \quad (1)$$

where k_{et} is the rate constant for electron transfer between cytochrome c_2 and cytochrome c-556 in the bound complex, and K_d is the dissociation constant (Strickland et al., 1975). The best fit was obtained with k_{et} = 270 s^{-1} and K_d = 30 μM. The extent of the re-reduction of cytochrome c-556 by cytochrome c_2 was 85% at a cytochrome c_2 concentration of 1 μM, and increased to 100% as the cytochrome c_2 concentration was increased to 40 μM. From these data, the difference between the redox potentials of cytochrome c-556 and cytochrome c_2 was estimated to be 0.03 ± 0.01 V. Double-flash experiments were carried out to determine how rapidly cytochrome c-556 could be sequentially re-reduced by two molecules of cytochrome c_2 (Figure 5A). The Phase-R laser was used for the first flash, the xenon flash lamp was used for the second flash, and the ubiquinone acceptor was 20 μM Q_{10}. The rate constants for reduction of cytochrome c-556 after the first and second flashes were 160 ± 20 and 150 ± 20 s^{-1}, respectively, for experiments with delay times ranging from 100 to 20 ms. There was no significant difference in the two rate constants for delay times as short as 20 ms. In another experiment, the delay between the two flashes was set to 1 ms in order to photooxidize both cytochrome c-556 and cytochrome c-559 before cytochrome c_2 could react (Figure 5B). The amplitude of the 559-nm absorbance transient following the double flash was 2.3 times as large as the 559-nm transient following a single saturating flash from the laser alone, indicating that the reduction of cytochrome c-559 contributes more than half of the amplitude of this transient. The transient could be fitted by a single first-order decay with a rate constant of 170 ± 15 s^{-1}. The rate constant for the 556-nm transient following either a double flash or a single laser flash was also 170 ± 15 s^{-1} for this sample (Figure 5B). These experiments indicate that cytochrome c-559 is reduced at the same rate as cytochrome c-556. Experiments carried out in the absence of ascorbate

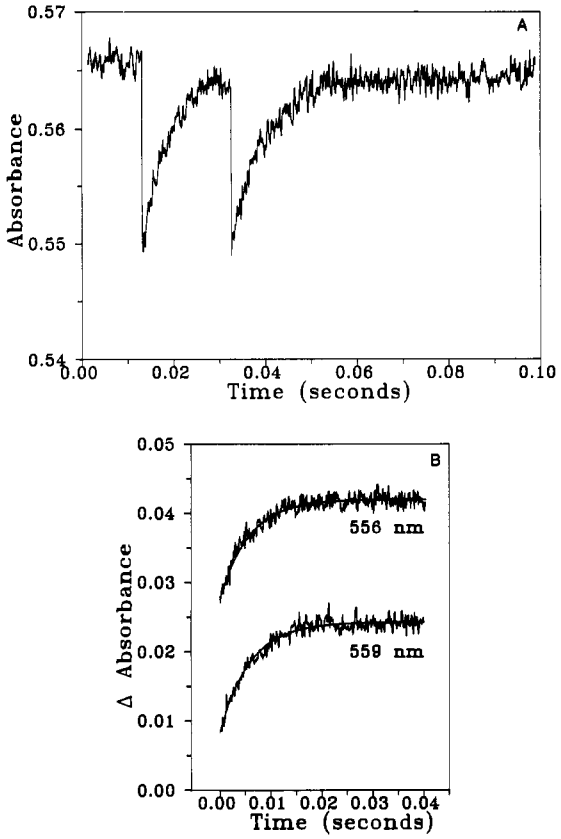

FIGURE 5: Double-flash kinetics of the reaction between cytochrome c_2 and cytochrome c-556. (A) The first flash was provided by a saturating 0.5-μs pulse-width laser beam, and the second by a saturating 100-μs xenon flash, with a delay time of 22 ms between the two flashes. The reaction mixture contained 1.5 μM purified reaction centers, 37 μM cytochrome c_2, 38 μM Q_{10}, and 1 mM ascorbate in 20 mM Tris-HCl, pH 8.0, containing 0.025% LDAO. The wavelength was 556 nm. (B) (Bottom) The 559-nm transient was recorded following a double flash with a delay time of 1 ms to photooxidize both cytochrome c-556 and cytochrome c-559. The solid line is the best fit to a single first-order decay with a rate constant of 170 s^{-1}. (Top) The 556-nm transient was recorded following a single laser flash to photooxidize only cytochrome c-556. The solid line has a rate constant of 170 s^{-1}. The reaction mixture was the same as in (A) above.

revealed that up to 30 μM excess oxidized cytochrome c_2 did not affect the rate of reduction of cytochrome c-556 by 5 μM ferrocytochrome c_2, indicating that the inhibition dissociation constant of oxidized cytochrome c_2 was greater than 50 μM (data not shown). The ionic strength dependence of the rate of the reaction between cytochrome c_2 and cytochrome c-556 is shown in Figure 6. The rate constant decreased by a factor of 14 as the ionic strength was increased from 5 mM to 1 M.

Photooxidation of Cytochrome c_2 by Rps. viridis Membrane Fragments. The photooxidation of cytochrome c_2 by reaction centers in membrane fragments isolated from *Rps. viridis* was similar to that observed using purified reaction centers (Figures 4 and 6). However, the extent of the reduction of photooxidized cytochrome c-556 by cytochrome c_2 was only 60% at 5 μM cytochrome c_2, increasing to 95% at 38 μM cytochrome c_2. The dependence of the rate constant on cytochrome c_2 concentration in 20 mM Tris-HCl was approximately linear up to 38 μM cytochrome c_2 (Figure 4). The ionic strength dependence of the rate constant was slightly smaller than observed with the purified reaction centers (Figure 6). Double-flash experiments revealed that there was no difference in the rates of cytochrome c_2 oxidation following the first laser flash and the second xenon flash, for delay times as short as 20 ms. The rate constant was also the same when the delay

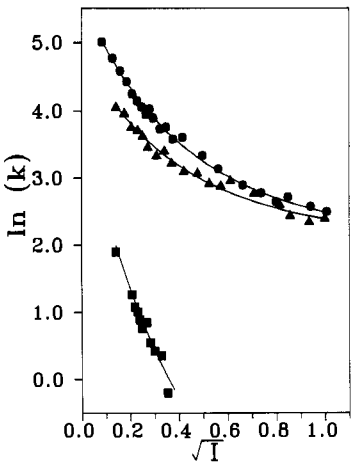

FIGURE 6: Ionic strength dependence of the single-flash kinetics of the reaction between cytochrome c_2 and purified reaction centers (●) or *Rps. viridis* membrane (▲). The observed first-order rate constant for the reaction of 44 μM cytochrome c_2 with purified reaction centers (●) or 38 μM cytochrome c_2 with membranes (▲) was measured as described in Figure 4 in buffers containing 0–1 M sodium chloride. The natural logarithm of the observed rate constant is plotted against $\sqrt{I}$. (■) The observed first-order rate constant for the reaction of 40 μM horse cytochrome c with 1 μM purified reaction centers as a function of ionic strength. The smooth curves are the best fits of eq 2 to the experimental data, as described in the text.

was decreased to 1 ms to initially photooxidize both cytochrome c-556 and cytochrome c-559.

Photooxidation of Horse Cytochrome c by Rps. viridis Reaction Centers. The oxidation of horse cytochrome c by *Rps. viridis* reaction centers was much slower than that of *Rps. viridis* cytochrome c_2. The reaction obeyed second-order kinetics up to a concentration of 30 μM, with a second-order rate constant of 1.7×10^5 M^{-1} s^{-1}. The observed rate constant was 5.6 s^{-1} at a concentration of 30 μM cytochrome c, compared to 130 s^{-1} for *Rps. viridis* cytochrome c_2 under the same conditions. The rate constant decreased by a factor of 8 as the ionic strength was increased from 15 to 120 mM (Figure 6).

Discussion

The kinetics of photooxidation of the cytochrome in the heme subunit of *Rps. viridis* reaction centers have been studied extensively by Dracheva et al. (1988), Shopes et al. (1987), and Kaminskaya et al. (1990). If both cytochromes c-556 and c-559 are initially reduced, then the first step is electron transfer from c-559 to P^+ with a half-time of 0.19 μs, followed by electron transfer from c-556 to c-559 with a half-time of 2.5 μs. Although the time resolution used in the present experiments was not sufficient to resolve these steps, the wavelength dependence of the rapid absorbance change between 0 and 25 μs after a single flash was the same as reported by Dracheva et al. (1988) and Shopes et al. (1987), indicating that cytochrome c-556 was photooxidized under these conditions. The slow reduction of cytochrome c-556 following photooxidation is probably mediated by absorbate present in the reaction mixture. Gao et al. (1990) reported that the rate constant for charge recombination of oxidized cytochrome c-556 with Q_A^- was 0.95 s^{-1} in the absence of ascorbate and the presence of o-phenanthroline to inhibit electron transfer from Q_A to Q_B. The charge recombination of cytochrome c-556 with Q_B^- is expected to be much slower, however. The additional photooxidation observed upon excitation with a second flash was due to oxidation of cytochrome c-559, as indicated by its wavelength dependence (Figure 2B). Since cytochrome c-556 was photooxidized by the first flash, it was

not available to rapidly reduce photooxidized cytochrome *c*-559 following the second flash. This additional photooxidation was dependent on the presence of added ubiquinone, since the purified reaction centers were depleted of natural ubiquinone Q_9 which normally occupies the Q_B site (Shopes & Wraight, 1985). When the short-chain nonphysiological ubiquinone Q_0 was used, the amplitude of the second phase was dependent on the delay time between the first and second flashes, probably because Q_0 did not support rapid electron transfer from Q_A to Q_B. However, when the long-chain ubiquinone Q_{10} was used, the amplitude of the second phase was independent of delay time down to 1 ms. In these experiments, it was necessary to use a laser flash with a pulse width of 0.5 μs for the first flash, since electron transfer from Q_A to Q_B could occur during a 25-μs xenon flash. Carithers and Parson (1975) have found that the half-time for electron transfer from Q_A^- to Q_B was about 30 μs in membranes containing the native ubiquinone Q_9.

Shill and Wood (1984) have carried out kinetic studies of a membrane fraction isolated from *Rps. viridis*, and found that one of the high-potential hemes in the reaction center cytochrome *c* subunit was reduced by soluble *Rps. viridis* cytochrome c_2, but did not identify which of the hemes was involved. They estimated a second-order rate constant of about 1×10^6 M^{-1} s^{-1} for this process but stated that the reaction was approaching saturation under the conditions used. The single-flash experiments reported in the present paper suggest that the reaction between cytochrome c_2 and cytochrome *c*-556 involves formation of an active complex according to the mechanism:

$$c_2^R + c_{556}^0 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} c_2^R\text{–}c_{556}^0 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} c_2^0\text{–}c_{556}^R \underset{k_6}{\overset{k_5}{\rightleftharpoons}} c_2^0 + c_{556}^R$$

If it is assumed that the dissociation rate constants k_2 and k_5 are fast compared to the electron-transfer rate constants k_3 and k_4, then k_{et} would be equal to k_3, while K_d would be equal to k_2/k_1 (Strickland et al., 1975). The hyperbolic dependence of the rate constant on cytochrome c_2 concentration (Figure 4) could be fitted with a dissociation constant K_d of 30 μM and a k_{et} value of 270 s^{-1}. However, alternative explanations of the hyperbolic dependence of the rate constant are possible, such as the presence of a slow, rate-limiting conformational change prior to the electron-transfer reaction. The extent of the reaction between reduced cytochrome c_2 and photooxidized cytochrome *c*-556 was consistent with a redox potential difference of 0.03 ± 0.01 V between the two cytochromes. This is in good agreement with the previously measured redox potentials, which are 285 mV for cytochrome c_2 (Pettigrew et al., 1978) and 312 mV for cytochrome *c*-556 (Shinkarev et al., 1990). Oxidized cytochrome c_2 did not inhibit the reaction of reduced cytochrome c_2 with cytochrome *c*-556, indicating that the dissociation constant of oxidized cytochrome c_2 was greater than 50 μM. It therefore appears that both reduced and oxidized cytochrome c_2 bind rather weakly to the reaction center.

The double-flash experiments demonstrated that cytochrome *c*-556 was reduced at the same rate after each of two flashes separated by as little as 20 ms. If it is assumed that there is a single site for reaction with cytochrome c_2, then the dissociation of cytochrome c_2 oxidized after the first flash must be fast enough to allow another molecule of reduced cytochrome c_2 to react with cytochrome *c*-556 photooxidized by the second flash. This assumption would require that k_5 must be greater than 100 s^{-1}. The experiment in which both cytochromes *c*-556 and *c*-559 were photooxidized by a double flash separated by 1 ms indicated that the rate of re-reduction of both cytochromes *c*-559 and *c*-556 was the same as the rate of re-reduction of cytochrome *c*-556 alone following a single laser flash. The simplest interpretation of this result is that cytochrome c_2 binds to a single site on the reaction center and reduces photooxidized cytochrome *c*-556 with a rate constant of 270 s^{-1}. Cytochrome *c*-556 then rapidly reduces cytochrome *c*-559 with a rate constant of 2.8×10^5 s^{-1} (Shopes et al., 1987), following which it is re-reduced by another molecule of cytochrome c_2. The observed rate of cytochrome *c*-559 reduction would be the same as that of cytochrome *c*-556 in this scheme, provided that the rate of dissociation of the first molecule of oxidized cytochrome c_2, k_5, was greater than about 200 s^{-1}. The results are not consistent with a mechanism in which cytochrome *c*-559 is directly reduced by cytochrome c_2 and subsequently reduces cytochrome *c*-556, since in that case the rate of reduction of cytochrome *c*-559 in the double-flash experiment would be much faster than that of cytochrome *c*-556 in the single-flash experiment.

The dependence of the observed rate constant on ionic strength suggests that electrostatic interactions contribute to the interaction of cytochrome c_2 with the reaction center. A semiempirical relation for the electrostatic interaction has been developed assuming that it can be represented by *n* specific charge pairs between lysine amino groups on cytochrome *c* and carboxylate groups on the partner (Smith et al., 1981). The ionic strength dependence of the rate constant is related to the electrostatic interaction of the *n* charge pairs by

$$\ln (k/k_\infty) = \sum_{i=1}^{n} -V_i/RT = \sum_{i=1}^{n} 4.235e^{\kappa(a-r_i)}/[RT(1 + \kappa a)r_i] \quad (2)$$

where V_i is the electrostatic energy of the *i*th charge pair, r_i is the distance between the amino and carboxylate groups of that charge pair, *a* is the effective radius of these groups, and $\kappa = 0.329\sqrt{I}$ $Å^{-1}$. Although this relationship is an oversimplification, it does provide a good empirical fit for a wide range of cytochrome *c* reactions, and allows comparisons to be made between the importance of electrostatic interactions to different reactions (Stonehuerner et al., 1979; Hall et al., 1987A). The value of *n* obtained by using this relation is in good agreement with chemical modification and molecular modeling studies of the complexes of cytochrome *c* with cytochrome b_5 and cytochrome *c* peroxidase (Stonehuerner et al., 1979; Smith & Millett, 1980). The best fit of this equation to the ionic strength dependence of the reaction between cytochrome c_2 and cytochrome *c*-556 was obtained by assuming that there are 3.5 charge pairs, $n = 3.5$, each with $r_i = 7$ Å (Figure 6). By comparison, the best fit of this equation to the reaction between *Rb. sphaeroides* cytochrome c_2 and *Rb. sphaeroides* reaction centers was obtained with $n = 4$ (Hall et al., 1987a). An *n* value of 5 provided a good fit for the reaction between *R. rubrum* cytochrome c_2 and *R. rubrum* reaction centers (Hall et al., 1987b).

The photooxidation of ferrocytochrome c_2 by membrane fragments isolated from *Rbs. viridis* was very similar to the reaction involving purified reaction centers. The major difference was that the dependence of the rate on cytochrome c_2 concentration was linear rather than hyperbolic, suggesting that the dissociation constant for the complex between cytochrome c_2 and the reaction center, K_d, was larger than 50 μM. However, it is not known whether the effective solution concentration of cytochrome c_2 near the reaction centers was the same as the total cytochrome c_2 added to the mixture. In fact, the extent of re-reduction of cytochrome *c*-556 by low concentrations of cytochrome c_2 was smaller in the membrane experiments than for the purified reaction centers. The re-

action centers might not be fully accessible to solution cytochrome c_2, or cytochrome c_2 might bind to additional sites on the membrane, lowering the effective solution concentration. A number of experiments have indicated that membranes isolated from *Rps. viridis* cells are in the form of flat lamellae rather than vesicles (Giesbrecht & Drews, 1966; Miller, 1982; Pucheu et al., 1973). This is consistent with the observation that photooxidized cytochrome *c*-556 is nearly completely re-reduced by cytochrome c_2 at high cytochrome c_2 concentrations. The dependence of the reaction rate on ionic strength was quite similar to that observed with purified reaction centers. The best fit of eq 2 to the data shown in Figure 6 was obtained with $n = 2.8$ and $r_i = 7$.

Horse cytochrome *c* displays considerable amino acid sequence homology and structural similarity to cytochrome c_2 from a number of purple bacteria. It is a highly effective surrogate for the native cytochrome c_2 in the reaction with reaction centers from *Rb. sphaeroides* (Hall et al., 1987a), *R. rubrum* (Hall et al., 1987b), and *Rhodocyclus gelatinosus* (Matsuura et al., 1988). It binds to *Rb. sphaeroides* reaction centers about 10 times more strongly than the native cytochrome c_2, and its rate of photooxidation is only about 10-fold slower (Overfield & Wraight, 1986). The sequence homology between horse cytochrome *c* and *Rps. viridis* cytochrome c_2 (Ambler et al., 1976) is actually larger than that between the horse and *Rb. sphaeroides* cytochromes (Dickerson, 1980). Four of the six lysine residues immediately surrounding the heme crevice of the horse protein are conserved in *Rps. viridis* cytochrome c_2. The large difference between the reaction rates of horse cytochrome *c* and *Rps. viridis* cytochrome c_2 with *Rps. viridis* reaction centers was therefore surprising. The best fit of eq 2 to the ionic strength dependence of the reaction of horse cytochrome *c* was obtained with $n = 6.5$ and $r = 6$ (Figure 6).

One of the most interesting questions raised by the present studies concerns the location of the cytochrome c_2 binding site on the *Rps. viridis* reaction center. The relatively slow rate for the reaction between cytochrome c_2 and cytochrome *c*-566 suggests that the cytochrome c_2 reaction site could be located some distance away on the heme subunit. As discussed above, the double-flash experiments indicate that the order of electron transfer could be cytochrome c_2 to *c*-556 to *c*-559, but not cytochrome c_2 to *c*-559 to *c*-556. This suggests that if there is a single reaction site for cytochrome c_2, it is probably located closer to *c*-556 than to *c*-559. The ionic strength dependence studies suggest that the interaction of *Rps. viridis* cytochrome c_2 with the reaction center involves a relatively small number of electrostatic interactions between lysine amino groups on cytochrome c_2 and carboxylate groups on the cytochrome subunit of the reaction center, possibly as few as three or four. The cytochrome *c*-556 heme group is nearly completely buried in the cytochrome subunit of the reaction center (Figue 1B). The only cluster of carboxylate residues on the surface of the cytochrome subunit is located near the cytochrome *c*-554 heme group, which is partially exposed to the surface of the molecule (Figure 1B). This cluster of carboxylate residues forms a ring around the exposed edge of the cytochrome *c*-554 heme group, with Asp-92 and Glu-93 to the immediate right of the heme, Glu-85 to the bottom right, Glu-79 to the bottom left, and Glu-67 to the top left (Figure 1B). This appears to be the most likely binding site for cytochrome c_2. All of the other carboxylate groups on the cytochrome subunit are located close to positively charged residues (Figure 1C). It is possible that cytochrome *c*-554 might facilitate electron transfer from cytochrome c_2 to cytochrome *c*-556, even though its redox potential is very low ($E_m < -50$ mV). Shopes et al. (1987) have suggested that cytochrome *c*-552 might function as a redox intermediate for electron transfer from cytochrome *c*-556 to cytochrome *c*-559, even though its redox potential is 300 mV lower than that of *c*-556 and *c*-559. The rather slow rate for electron transfer between cytochrome c_2 and cytochrome *c*-556 is consistent with an equivalent role for cytochrome *c*-554.

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Registry No. Cytochrome c_2, 9035-43-2; cytochrome *c*, 9007-43-6; cytochrome *c*-556, 37306-15-3; cytochrome *c*-554, 52932-67-9; glutamate, 56-86-0.

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Solution Structure of the Basic Region from the Transcriptional Activator GCN4

V. Saudek,[‡] H. S. Pasley,[‡] T. Gibson,[§] H. Gausepohl,[§] R. Frank,[§] and A. Pastore*[,§]

Merrell Dow Research Institute, 16 rue d'Ankara, B.P. 447/R9, 67009 Strasbourg, France, and European Molecular Biology Laboratory, Heidelberg, Germany

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ABSTRACT: The structure of the basic region (i.e., the region responsible for sequence-specific binding to DNA) of the transcriptional activator GCN4 was studied. Two peptide fragments containing either the basic region alone (residues 240–280) or the basic and the dimerization leucine zipper domains (220–280) were synthesized and investigated by nuclear magnetic resonance and circular dichroic spectroscopy. The basic region in the absence of DNA appears as a mobile flexible segment folded into a loose helix. The helical stability increases upon addition of trifluoroethanol and/or lowering of the temperature. Dimerization via the leucine zipper does not affect the three-dimensional structure of the basic region. Possible consequences for the binding to DNA are discussed.

A class of transcriptional activators called the "leucine zipper" family has recently been discovered [Landschulz et al., 1988; for a review see, e.g., Busch and Sassone-Corsi (1990)]. These proteins are active as dimers and contain two functionally distinct regions necessary for DNA binding: One (the leucine zipper domain) is responsible for the formation of the active dimer. The other (basic region) is required for the sequence-specific binding to DNA (Hope & Struhl, 1987).

Considerable evidence, coming from molecular biology (Turner & Tjian, 1989), chemical studies (O'Shea et al., 1989), secondary structure predictions (Busch & Sassone-Corsi, 1990), and circular dichroic spectroscopy (O'Shea et al., 1989), indicates that the conformation of the leucine zippers is an amphiphilic α-helix. It was suggested that the dimerization was based on the hydrophobic interaction of the side chains and the arrangement of the helices was head to head, probably forming a coiled coil (O'Shea et al., 1989). A so-called "scissors-grip" model of the structure has been presented (Vinson et al., 1989), where the leucine zippers bring together the adjacent basic regions such that they can wrap around the DNA in the major groove. According to this model, the basic regions are also folded predominantly in an α-helical conformation which is broken at an asparagine residue.

An interesting representative of this family of transcriptional activators is GCN4, a yeast protein whose sequence was de-

* To whom correspondence should be addressed.
‡ Merrell Dow Research Institute.
§ European Molecular Biology Laboratory.

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