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EQUILIBRIUM AND KINETIC MEASUREMENTS OF THE REDOX POTENTIALS OF CYTOCHROMES c_2 IN VITRO AND IN VIVO

ROGER C. PRINCE and C. LINDSAY BASHFORD

Johnson Research Foundation and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104 (U.S.A.)

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Summary

The equilibrium oxidation-reduction mipoint potential (E_m) of isolated *Rhodopseudomonas sphaeroides* cytochrome c_2 exhibits a pH-dependent behavior which can be ascribed to a pK on the oxidized form at pH 8.0 (Pettigrew et al. (1975) Biochim. Biophys. Acta 430, 197—208). However, as with mammalian cytochrome *c* (Brandt, K.G., Parks, P.C., Czerlinski, G.H. and Hess, G.P. (1966) J. Biol. Chem. 241, 4180—4185) this pK can more properly be attributed to the combination of a pK beyond pH 11, and a slow conformational change of the ferricytochrome. This has been demonstrated by resolving the E_m of cytochrome c_2 before and after the conformational change. The E_m of the unaltered form is essentially pH independent between pH 7 and 11.5, and the lower equilibrium E_m is due solely to the conformational change. In vivo the conformational change is prevented by the binding of the cytochrome c_2 to the photochemical reaction center, and the cytochrome exhibits an essentially pH-independent E_m from pH 5 to 11. The alkaline transition thus has little physiological significance, and it is unlikely that the redox reactions of cytochrome c_2 in vivo involve protons.

Introduction

Cytochromes c_2 are a class of *c*-type cytochromes found in the non-sulfur purple bacteria (Rhodospirillaceae). In many species they serve as the immediate electron donor to the photochemical reaction center, or to a terminal oxidase of the respiratory chain, depending on the mode of growth, but in other species no clear role has been assigned [1,2]. Both the amino acid

sequences and the morphologies of the heme-binding sites of cytochromes c_2 are very similar to those of mammalian cytochromes *c* [3,4], but the redox potentials at neutral pH are somewhat more positive [5]. Pettigrew et al. [5] have recently examined the pH dependency of the midpoint potentials of many of the cytochromes c_2, ascribing the dramatic pH dependence at alkaline pH to a p*K* on the oxidized form in the range pH 7—9.5. Several cytochromes also exhibited ionizations on both the oxidized and reduced forms near neutral pH [5,6]. In contrast, titrations of the cytochrome c_2 which is rapidly oxidized by the reaction center in vivo reveal no significant pH dependency of the midpoint potential between pH 5 and 11 [7].

In this paper we re-investigate the pH dependency of the mipoint potential of cytochrome c_2 from *Rhodopseudomonas sphaeroides*. In vitro this cytochrome has many properties analogous to those of mammalian cytochrome *c*. An elegant series of experiments by Brandt et al. [8] and Davis et al. [9] has demonstrated that ferricytochrome *c* undergoes a conformational change at alkaline pH which depends on the ionization of a group with a p*K* which they predicted to be near 11. The combination of the conformational change with the ionization leads to an apparent p*K* for the alkaline transition of ferricytochrome *c* around pH 9. In the experiments reported here we demonstrate that the p*K* on *Rps. sphaeroides* ferricytochrome c_2 is indeed beyond pH 11, although the subsequent conformational change shifts the p*K* to pH 8 [6]. In vivo the cytochromes c_2 free in solution can be resolved thermodynamically from those bound to the reaction center at the membrane aqueous interface. The former exhibit the pH-dependent midpoint potential described by Pettigrew et al. [5,6], the latter the pH-independent E_m reported by us earlier [7]. The addition of cholate converts all the cytochrome to the in vitro, pH-dependent form. A preliminary account of part of this work has been presented [10,11].

Materials and Methods

Rps. sphaeroides Ga, *Rhodopseudomonas capsulata* St. Louis, *Rhodopseudomonas viridis* NHTC 133, and *Rhodospirillum rubrum* S1 were grown photosynthetically with succinate as sole carbon source, and chromatophores were prepared as described earlier [12]. Cytochromes c_2 from the respective organisms were prepared according to the method of Bartsch [13]. The kinetics of the conformational change were measured in an Aminco-Chance dual wavelength/split beam recording spectrophotometer, operating in the split beam mode, or in a Perkin-Elmer 557 double-wavelength, double-beam spectrophotometer equipped with a Union Giken MX-7 stopped flow attachment. The E_h and pH of the effluent of the flow cell were measured at the completion of each reaction.

Results

In vitro

Fig. 1 shows the reduction of cytochromes c_2 under three different conditions. Trace c represents the reduction of the ferricytochrome by ferrocyanide

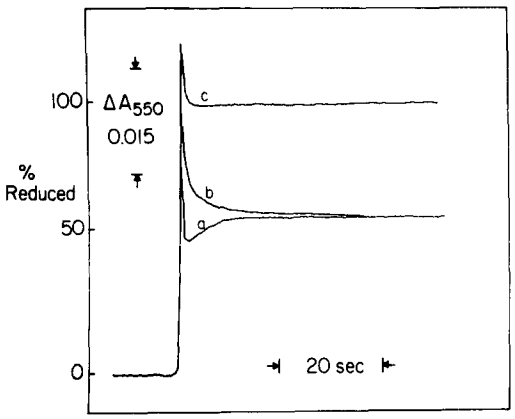

Fig. 1. The reduction of ferricytochrome c_2 by ferrocyanide. The reduction of 2 μM *Rps. sphaeroides* ferricytochrome c_2 by 200 μM $K_3Fe(CN)_6/K_4Fe(CN)_6$, E_h of 252 mV (a) at pH 10.0; (b) pH 7.1 and basic ferrocyanide to yield a final pH of 10.0, and (c) pH 7.1. The buffer was 10 mM glycine, 100 mM KCl, pH 10.0, or 10 mM *N*-morpholinopropane sulfonate, 100 mM KCl, pH 7.1.

at pH 7, while trace a is a repeat of the experiment at pH 10. As expected from the data of Pettigrew et al. [5,6] and Davis et al. [9], the extent of ferricytochrome reduction by the ferri/ferrocyanide mixture (E_h 252 mV) is substantially less at pH 10 than it is at pH 7, because the equilibrium E_m of the cytochromes is significantly more negative at the alkaline pH. The biphasic kinetics of cytochrome c_2 reduction at alkaline pH (Fig. 1, trace a) are readily apparent, with the slow phase having a time course of several seconds. If the experiment is repeated (trace b) so that the ferricytochrome is equilibrated at pH 7, but the ferrocyanide addition also raises the pH, a quite different trace is obtained. On the time scale of Fig. 1, the alkaline ferrocyanide causes a transient reduction of the cytochrome, followed by a slow re-oxidation to the level of reduction seen if the cytochrome were pre-equilibrated at pH 10. We have determined the

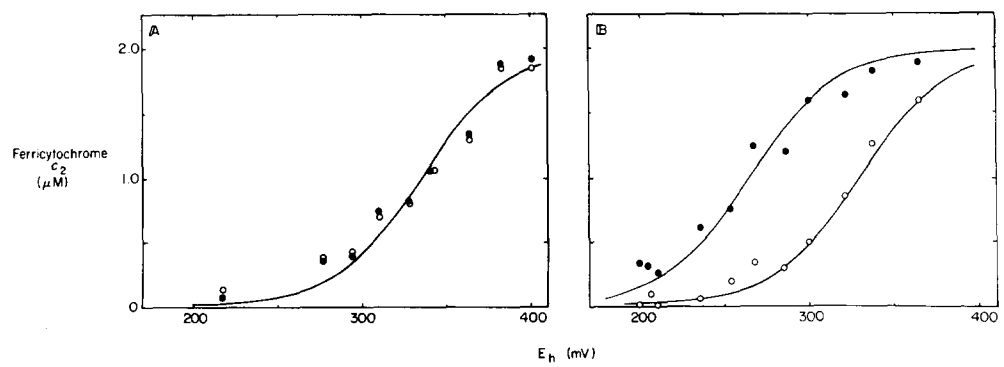

Fig. 2. Redox titration of cytochrome c_2. *Rps. sphaeroides* ferricytochrome c_2 at pH 7 was added to 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ in 10 mM *N*-morpholinopropane sulfonate, pH 6.8 (A), or 10 mM glycine, pH 10 (B), to yield a final concentration of 2 μM. The concentration of ferricytochrome was determined at the time of mixing (○) and at equilibrium (●).

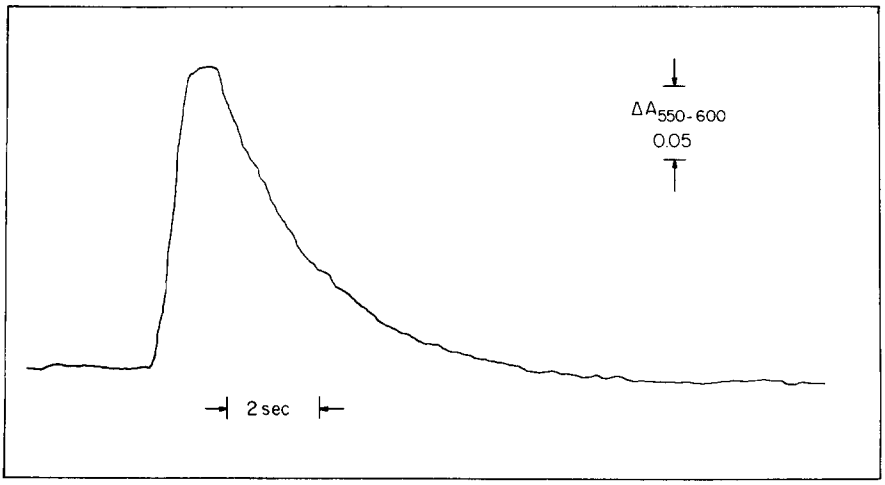

Fig. 3. The reduction of *Rps. sphaeroides* ferricytochrome c_2 by ferrocyanide. 1 ml of 30 μM ferricytochrome c_2, pH 7 was mixed with 1 ml of 100 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ in 20 mM arginine to yield a final pH of 11.6 and E_h of 247 mV.

amount of ferricytochrome present at the time of mixing and 2 min later, and have repeated the experiments with a variety of mixtures of ferri/ferrocyanide to yield a redox titration. The results are shown in Fig. 2. At pH 7, the E_m of cytochrome c_2 is the same whether measured at the time of the mixing, or 2 min later, but at pH 10 there is a significant difference between the E_m values measured at the two times. The E_m measured at the time of mixing is only 10 mV less than that measured at pH 7, but after 2 min the E_m has dropped 70—260 mV. Even at pH 11.5 where the conformational change is sufficiently fast that rapid mixing in a stopped-flow apparatus is required to resolve the ferricytochrome concentration at the time of mixing (Fig. 3), the E_m at the time of mixing is 310 mV, while within 2 min it drops to 160 mV. The data from similar experiments at a variety of pH values are collated in Fig. 6.

The drop in E_m which occurs at alkaline pH is readily reversed when the pH is returned to neutrality (Fig. 4). Typical half-times for the conformational change following the base pulse to pH 10, and the acid pulse to pH 7, were 3 s and 10 s, respectively, for all the bacterial cytochromes c_2 listed in Materials and Methods. These values are in remarkable agreement with those of Davis et al. [9] and Kihara et al. [14] for equine cytochrome *c*.

In vivo

In a previous paper, we have shown that the cytochrome c_2 rapidly oxidized by the photochemical reaction center after a flash of light exhibits a pH-independent midpoint potential between pH 5 and 11. However, during the preparation of chromatophores, some 30—60% of the reaction centers lose their cytochrome c_2, which is then presumeably released into the chromatophore interior [11,15]. Dutton et al. [12] showed that this free cytochrome, which is not rapidly oxidized by the photochemical reaction center, has an equilibrium E_m at pH 7 of 340 mV instead of the 295 mV measured when it is attached to

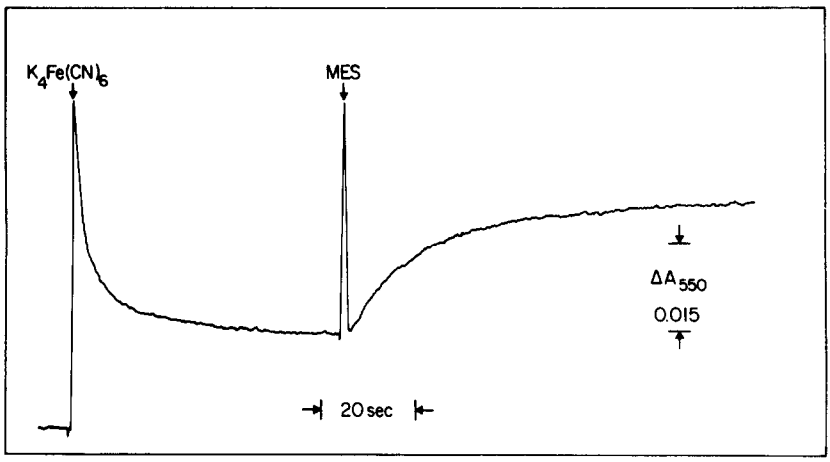

Fig. 4. The reversibility of the E_m shift. 30 µl of 8 mM $K_4Fe(CN)_6$ in 100 mM glycine (pH 10.8) was added to 3.0 ml of 2 µM *Rps. sphaeroides* ferricytochrome c_2 in 60 mM K_2SO_4/2 mM ethylenedinitrilodiamine tetraacetate, pH 7.0, bringing the pH to 9.9. 30 µl of 500 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.0, was then added to bring the pH to 7.1.

the reaction center. The addition of cholate liberates all the cytochrome c_2, which then exhibits a single E_m at pH 7 of 340 mV [15]. We have repeated these experiments at pH 10.2 (Fig. 5) and, in the absence of detergent, the redox titration of cytochrome c_2, measured on a scanning double-beam spectrophotometer, reveals that 60% of the cytochrome has an E_m of 290 mV, while 40% has an E_m of 210 mV, attributable to the bound and free cytochrome,

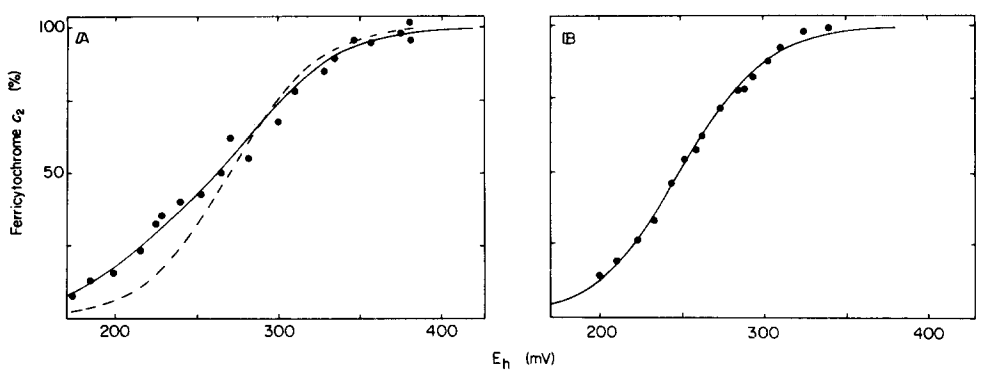

Fig. 5. Redox titrations of cytochrome c_2 in the chromatophore. *Rps. sphaeroides* Ga chromatophores (46 µM bacteriochlorophyll, 1.1 µM cytochrome c_2) were suspended in 10 mM glycine, 100 mM KCl, 1 mM $MgCl_2$, 20 µM 2,3,5,6-tetramethylphenylenediamine, *N*-methyl phenazonium methosulfonate and *N*-ethyl phenazonium ethosulfate. In (A) the pH was 10.2; ———, the theoretical curve for 39% of the cytochrome having an E_m of 210 mV and 61% having an E_m of 290 mV. - - - - - -, the theoretical curve expected if there were only one population of cytochrome, with an E_m of 270 mV. (B) The pH was 9.8, and 1% cholate was present. The line is the theoretical curve for all the cytochrome having an E_m of 247 mV.

respectively. The addition of cholate liberates all the cytochrome, which then assumes a single equilibrium E_m of 247 mV at pH 9.8.

Discussion

The alkaline transition of cytochrome c_2

The pH dependence of the oxidation reduction midpoint potential of *Rps. sphaeroides* cytochrome c_2 is shown in Fig. 6, which combines the data of Pettigrew et al. [5,6], Prince and Dutton [7], Dutton et al. [12], Prince et al. [15] and the experiments reported here. On the left of Fig. 6 is the data for isolated cytochrome c_2 in solution, together with the data for what is believed to be cytochrome c_2 in solution within the chromatophore (Ref. 12 and Fig. 5). It is clear from Fig. 6 that there is excellent agreement in the equilibrium values for the E_m of cytochrome c_2, which, as pointed out be Pettigrew et al. [5,6], can be ascribed to apparent pK values on the oxidized cytochrome at pH 6.1 and pH 8.0 and on the reduced form at pH 7.4. However, the pK at pH 8 is only an apparent pK, since it is measured after the 'alkaline transition'. The E_m measured at the time of mixing neutral ferricytochrome with alkaline ferrocyanide shows little pH dependence, as predicted by Brandt et al. [8] and Davis

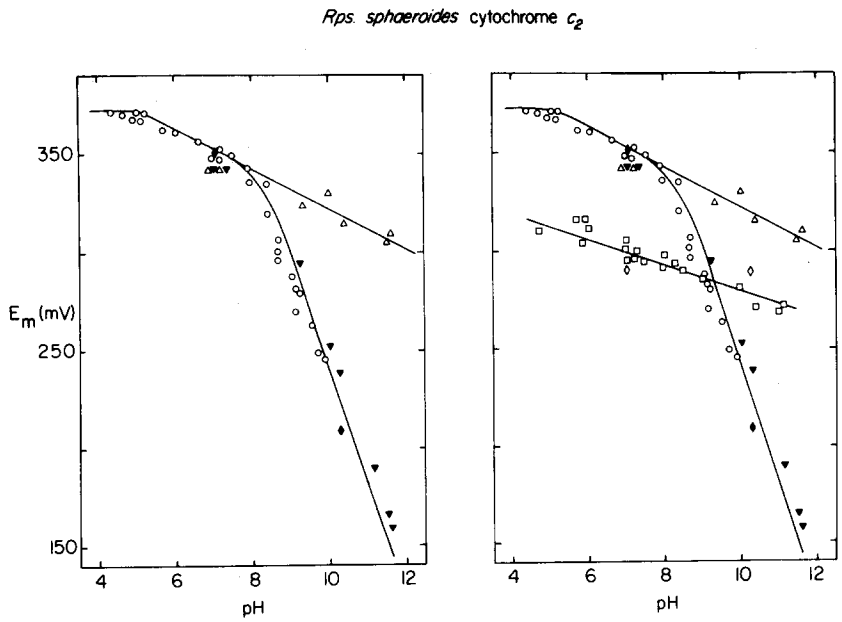

Fig. 6. The pH dependency of the E_m of *Rps. sphaeroides* cytochrome c_2. On the left is the data for cytochrome c_2 in solution. ○, data from Pettigrew et al. [5]; △, ▲, data of this paper. △, E_m at the time of mixing neutral ferricytochrome with alkaline ferrocyanide; ▲, E_m 2 min later. ♦, data for the cytochrome c_2 believed to be in solution inside the chromatophore, unattached to the reaction center (Ref. 12 and Fig. 4). On the left, the data for the cytochrome c_2 attached to the reaction center in the chromatophore is superimposed upon the data in solution. □, data of Prince and Dutton [7], obtained by monitoring the extent of rapid (millisecond) photooxidation after a single turnover flash. ◇, E_m values for the reaction center-associated cytochrome c_2 obtained at equilibrium (Ref. 12 and Fig. 4).

et al. [9], and the data indicate that there is no significant pK for the unaltered cytochrome below pH 11.

The alkaline transition of mammalian ferricytochrome c has been a much studied phenomenon, and it is now known that at alkaline pH the ferricytochrome loses an absorption band at 695 nm, ascribed to a charge-transfer band [17], and a proton [8,9,14,17—20]. Stopped-flow experiments have revealed at least two pK transitions beyond pH 10 [14,15], but the simpler scheme originally proposed by Brandt et al. [8], involving only a single pK, is adequate to explain the data for cytochrome c_2 presented here. The model of Brandt et al. [8] invokes a pK near pH 11 on the oxidized form. The deprotonated ferricytochrome still has a 695 nm band, but can undergo a rather slow conformational change to a form which lacks the 695 nm band [8,9]. The equilibrium constant for the conformational change shifts the pK from its predicted values near 11 to an apparent value near 9 by removing the deprotonated form from the protolytic equilibrium [8,9]. The conformational change is sufficiently slow (k in the range 0.07—0.3 s^{-1} [9,14], and see Figs. 1, 3 and 4) that a redox equilibrium with ferri/ferrocyanide mixtures can be achieved both before and after the conformational change. The slow phases of oxidation and reduction (Figs. 1, 3 and 4, and cf. Refs. 20—22) are limited by the rate of the conformational change, and their amplitude is governed by the apparent pK. Only after the conformational change has occurred does the E_m drop so as to yield the lower pK, and it is clear that changing the equilibrium constant(s) for the conformational change(s) would alter the apparent pK. Such effects, based on subtle differences in the structures of cytochrome c_2 from different species, would explain the considerable variation of the apparent pK seen in the photosynthetic bacteria [5,6], and it may be premature to assign the pK to specific groups near the heme cleft [5], or in the heme itself [6].

The behavior of cytochrome c_2 in vivo

Cytochrome c_2 attached to the reaction center of *Rps. sphaeroides* does not exhibit a pK on the ferricytochrome in the range pH 5—11 (Ref. 7, Fig. 6), suggesting that the alkaline transition is prevented by the interaction of the cytochrome with the reaction center. This interaction also lowers the equilibrium E_m of the bound cytochrome by 45 mV at pH 7 [12,15]. In contrast, the E_m of the free cytochrome within the chromatophore, which was presumably dislodged during preparation, clearly exhibits the pH dependence of isolated cytochrome c_2 in vitro (Refs. 5 and 6, and see Figs. 5 and 6). Thus it is clear that cytochrome c_2 in its functional location at the membrane-aqueous interface [15] does not undergo any net changes in protonation during its interaction with the reaction center (see Ref. 12) or its reductant in the ubiquinone-cytochrome b-c_2 oxidoreductase (see Ref. 23).

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