CONFORMATION CHANGES IN CYTOCHROME c_2 FROM *RHODOSPIRILLUM RUBRUM*

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1. Introduction

The tertiary structure of cytochrome c_2 from *Rhodospirillum rubrum* [1] is closely related to that of horse cytochrome *c* [2,3]. The ligands of the iron, the structure of the haem crevice and the characteristic arrangement of aromatic groups are common to both proteins. However, cytochrome c_2 has different enzymic properties [4] and certain features of the tertiary structure are different, notably the behaviour of phenylalanine residue 93 (corresponding to Phe 82 in horse cytochrome *c*) which is situated inside the haem crevice in both the oxidized and reduced crystal forms. Thus the large movement of the peptide chain including Phe 82 which occurs during the oxidation and reduction of horse cytochrome *c* does not occur in the equivalent region of cytochrome c_2. Certain physico-chemical properties might be expected to be altered as a result of this.

2. Materials and methods

Cytochrome c_2 from *Rhodospirillum rubrum* was a kind gift from Dr. R. G. Bartsch through the good offices of Drs. T. E. Meyer and R. P. Amber. We are indebted to them for this gift.

2.1. *Variation of the redox potential with pH*

The variation of the redox potential of cytochrome c_2 with pH was investigated spectrophotometrically using 2 mM Tris–HCl buffers between pH 7.5 and 9.0 and 2 mM carbonate–bicarbonate buffers between pH 9.0 and 10.6.

2.2. *Spectrophotometric titration at alkaline pH*

Difference spectra in the region of the Soret band and absolute spectra between 670 and 730 nm were used to study the spectroscopic changes which occurred during titration at alkaline pH. Appropriate small additions of NaOH solution were made to an unbuffered solution of cytochrome at pH 7.0 and the pH and the spectrum were recorded after each addition.

2.3. *Rapid kinetics*

The reduction of cytochrome c_2 by dithionite at alkaline pH was examined using a Durrum stopped-flow apparatus. Protein was dissolved in 0.2 M carbonate–bicarbonate buffer pH 10.5 or 0.2 M Na_2HPO_4–NaOH buffer pH 11.5. Fifty sodium dithionite was prepared by dissolving the solid in the appropriate buffer and readjusting the pH.

3. Results

A p*K* of 9.2 was estimated for the disappearance of the 697 nm band (fig. 1). In the Soret difference spectra there were at least two spectroscopically operable p*K*'s between pH 8 and 12 (fig. 1). The titration of the second p*K* involved a shift in the peak of the difference spectrum from 404 to 402 nm. Approximate p*K* values of 9 and 11 were estimated.

A single fast reaction was observed in the reduction of cytochrome c_2 with dithionite at pH 10.5. At pH 11.5 a second slow change was observed involving a decrease in extinction at 550 nm and with first-order rate constant of 6.1 sec^{-1} which was independent of concentration of reductant. Under the same

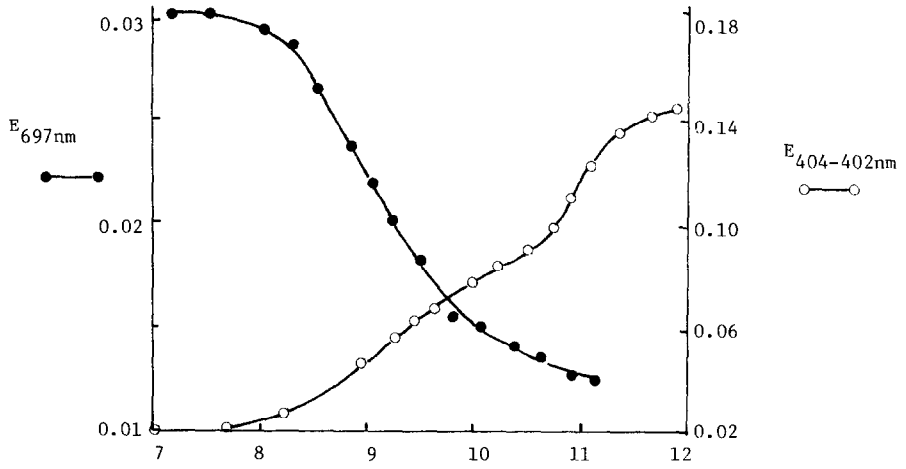

Fig. 1. Spectrophotometric titration of cytochrome c_2. (●——●) Plot of extinction at 697 nm against pH (cytochrome, 4×10^{-6} M) (○——○) Plot of extinction at 404–402 nm against pH (cytochrome, 5×10^{-5} M) Data are from difference spectra (pH 6.5 v pH alkaline) and the position of the peak varies with pH between 402 and 404 nm.

conditions the rate constant for the slow reaction of cytochrome *c* was found to be 2.8 sec^{-1} at pH 10.5 and 1.8 sec^{-1} at pH 11.5 in agreement with the figure of 2 sec^{-1} found by Lambeth et al. [5] for pH 11.

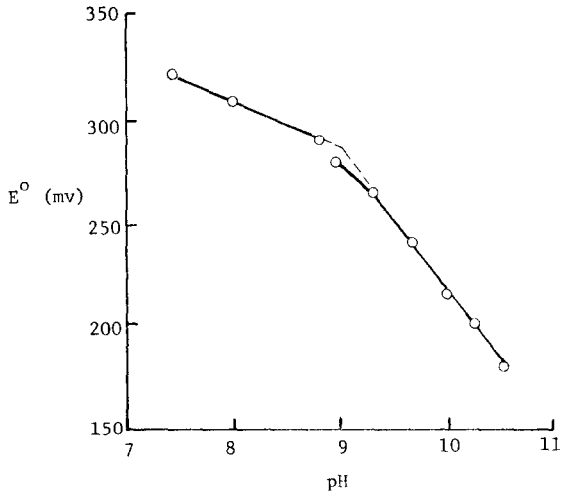

Fig. 2. Variation of the redox potential of cytochrome c_2 with pH. Cytochrome concentration was 1×10^{-5} M in 0.002 M Tris–HCl or carbonate–bicarbonate buffers of varying pH. 250 nmoles ferrocyanide were added up to pH 9.1 and 500 nmoles thereafter.

The variation of the redox potential with pH is shown in fig. 2. The results of Kamen and Vernon [6] were therefore confirmed for pH 7.0 to 9.0 but above pH 9.0 the redox potential falls rapidly in an analogous fashion to cytochrome *c* [7,8]. The slope of 70 mV/pH unit is consistent with a single proton dissociation and the estimated p*K* is approximately 9.3. This is very similar to the results for cytochrome *c* and in contrast to the small decrease in the redox potential of cytochrome c_2 from 380 mv at pH 5 to 300 mV at pH 8.0 [6].

4. Discussion

The X-ray studies on cytochrome c_2 [1] have shown that the molecule has a structure in both oxidized and reduced states most resembling ferrocytochrome *c*. In particular the phenylalanine residue at position 93 (corresponding to Phe-82 in cytochrome *c*) is positioned inside the haem pocket in both oxidation states. Because of this it might be expected that cytochrome c_2 should show no haem-linked alkaline p*K* (like the reduced form of cytochrome *c*) and that reduction at alkaline pH with dithionite might proceed through a monophasic process, without the second slow conformation change observed in cytochrome *c*.

In addition it is possible that cyanide reacts with ferricytochrome *c* by displacing Met-80 into the space occupied by Phe-82 in the reduced protein. This would explain why cyanide does not form a stable complex with ferrocytochrome *c* and by analogy ferricytochrome c_2 should not react with cyanide. Finally, the small conformational changes occuring on oxidation and reduction of cytochrome c_2 may be reflected in the thermodynamic parameters of this reaction.

These expectations were not fulfilled. Horio and Kamen [9] showed that the spectrum of ferricytochrome *c* does change in alkaline pH. We found that the 697 nm band disappears in a single step ionisation with an approx. p*K* of 9. This was accompanied by a decrease in the redox potential, also with an approximate p*K* of 9. These results are very similar to the results for cytochrome *c* and are assumed to reflect a breaking of the methionine–iron coordination.

Two observations, however, distinguish cytochrome c_2 from cytochrome *c*. In cytochrome c_2 changes are observed in the Soret band with rising pH after the titration of the 697 nm band is complete. This indicates that a second haem linked ionization occurs in cytochrome c_2 which does not occur in cytochrome *c* (or, at least, is not haem-linked and spectroscopically observable). Interestingly, Gupta and Koenig [10] have reported that two p*K*'s can be detected in the NMR spectra of cytochrome *c* at alkaline pH. They show that the p*K* near 9 is the breaking of the methionine–iron bond and suggest that it is the consequence of deprotonation of Lys-79. These authors find a second p*K* near 11 like the p*K* found in this work for cytochrome c_2.

Secondly, unlike cytochrome *c*, cytochrome c_2 does not undergo a slow relaxation after reduction with dithionite at alkaline pH's up to 10.5. In the case of cytochrome *c* this slow change is interpreted as a replacement of the ligand of the iron by methionine 80 [5]. Lysine-79 is the proposed 6th ligand for ferricytochrome *c* and, for the ferrocytochrome *c* transient at alkaline pH. Cytochrome c_2 does undergo a slow relaxation at pH 11.5 and this may be linked with the spectroscopically operable p*K* 11 of the oxidized form.

These results present a problem in interpretation. If the current belief that the slow conformational change on reduction is a replacement of lysine by methionine as the 6th ligand of Fe^{2+}, is correct then, by analogy ferricytochrome c_2 does not have lysine as the 6th ligand until above pH 10.5. This, however, leaves the problem of the nature of the iron coordination in the pH range between the first p*K* and 10.5 as the first p*K* appears to be consistent with the loss of methionine coordination.

An alternative explanation is that the p*K* of 9 in ferricytochrome c_2 reflects a ligand change without much conformational change, perhaps because the methionine cannot be displaced into the pocket which contains Phe-93 in both the reduced and oxidized forms. The second p*K* may then reflect a conformation change in the oxidized protein which is reversed on reduction at this alkaline pH.

Contrary to expectations and the statement of Krejcorek et al. [11] that cytochrome c_2 does not bind cyanide, we found that the 697 nm band was abolished by 0.025 M KCN at pH 7.0. Reduction of the cyano-cytochrome c_2 with dithionite gave rise to a short-lived species with α-peak shifted approx. 5 nm to the red and which decayed to give a normal spectrum in a few minutes at 15°C. These results are qualitatively similar to the reaction cytochrome c with cyanide [12,13]. Thus even though Phe 93 sits inside the crevice of cytochrome c_2, cyanide can still gain access to the ferrihaem and displace the methionine under the conditions used.

References

[1] Salemme, R. R., Kraut, J. and Kamen, M. D. (1973) J. Biol. Chem. 248, 7701–7716.
[2] Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A. and Margoliash, E. (1971) J. Biol. Chem. 246, 1511–1535.
[3] Takano, T., Kallai, O. B., Swanson, R. and Dickerson, R. E. (1973) J. Biol. Chem. 248, 5234–5244.
[4] Davis, K., Hatefi, Y., Salemme, F. R. and Kamen, M. D. (1972) Biochem. Biophys. Res. Commun. 49, 1329–1335.
[5] Lambeth, D. O., Campbell, K. L., Zand, R. and Palmer, G. (1973) J. Biol. Chem. 248, 8130–8136.
[6] Kamen, M. D. and Vernon, L. P. (1955) Biochim. Biophys. Acta 17, 10–12.
[7] Rodkey, F. L. and Ball, E. G. (1950) J. Biol. Chem. 182, 17–28.
[8] Margalit, R. and Schejter, A. (1973) Eur. J. Biochem. 32, 492–499.

[9] Horio, T. and Kamen, M. D. (1961) Biochim. Biophys. Acta 48, 266–286.
[10] Gupta, R. K. and Koenig, S. H. (1971) Biochem. Biophys. Res. Commun. 45, 1134–1143.
[11] Krejcorek, G. E., Turner, L. and Dus, K. (1971) Biochem. Biophys. Res. Commun. 42, 983–991.
[12] George, P., Glauser, S. C. and Schejter, A. (1966) J. Biol. Chem. 242, 1690–1695.
[13] George, P. and Schejter, A. (1964) J. Biol. Chem. 239, 1504–1508.