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CONFORMATION-DEPENDENT PARTICIPATION OF THE PROTEIN IN ELECTRON EQUIVALENT TRANSFER TO CYTOCHROME *c*

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SUMMARY

When ferricytochrome *c* is reduced by H atoms (produced by pulse radiolysis) at neutral pH where it is in a closed protein configuration, a considerable percentage of the reduction proceeds through electron equivalent transfer via the protein.

At pH 2.0, where cytochrome *c* is in an open configuration, H atoms reduce by adding directly to the heme porphyrin. The intermediate then observed is identified through similarity with that formed on ferriheme alone.

INTRODUCTION

Do organic macromolecules in biological systems, particularly proteins as distinct from metal-containing components, actively participate in conveying electron equivalents in redox reactions? If they do, by what mechanisms? This central question of bioenergetics has frequently been discussed and those favoring a positive answer proposed in some cases mechanisms involving tunneling or intramolecular free-radical transfer as possible interpretations of some experimental facts. Clear demonstrations of protein moiety participation in redox processes remain to be desired.

The protein polypeptide chain of the electron carrier enzyme cytochrome *c* [1] is covalently bound to the heme. When the enzyme is reduced the oxidation state of its heme-iron changes from Fe(III) to Fe(II). Extensive studies on the physicochemical [2] and structural [3, 4] properties of CIII and CII led to two possible mechanisms for its reduction. In one, the region of the heme site itself is believed to be the key point for interaction in the electron equivalent transfer [5–7]; in the other, the polypeptide chain of the protein moiety is believed to play an active role in the transfer of the electron equivalent to and from the metal prosthetic group [8]. Recently the second mechanism has been subjected to severe critical re-evaluation [1] and doubts expressed whether it does operate.

We report our results on the kinetics of the reduction of ferricytochrome *c* (CIII) by H atoms using the fast-kinetic spectroscopy-pulse radiolysis technique [9]

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on a time scale extending from approx. 100 ns to seconds. Our results show, for this very reactive, non-physiological reagent, two reaction pathways depending on the pH, which affects the conformation of the protein moiety of the metallo-enzyme. In one, where the protein at neutral pH is in the close conformation, an appreciable part of the reduction proceeds via the protein. In the other, at acid pH, where the open conformation of the protein permits easier direct access to the iron porphyrin prosthetic group, protein participation is not apparent and direct interaction with the heme region prevails.

Previous studies of such reduction near neutral pH by hydrated electrons, e_{aq}^-, [10–15] showed that all e_{aq}^- produced react rapidly with CIII, but only some 70–80 % of these cause reduction of CIII to CII [14, 15]. The rest probably form peripheral radical adducts on the protein, which end up without reducing ferri to ferro. The mechanism of the observed 70–80 % reduction of native CIII at neutral pH by e_{aq}^- may involve electron transfer processes through the protein as well as more direct reaction of this reducing substrate with the heme region [14, 15]. A clear separation between the two mechanisms could not be obtained. The very fast diffusion limited bimolecular reduction ($k = 2 \cdot 10^{10}$–$6 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ at pH ≈ 7, dependent on ionic strength) by hydrated electron is followed by two slower intramolecular processes [14, 15] with first-order rate constants of approx. 10^5 and approx. $10^2\ s^{-1}$. These are probably due to conformational changes. For example, experiments from 5 to 35 °C on the temperature dependence of these gave activation energies of 7.5 and 14 kcal/mol, respectively, for the fast and slow first-order intramolecular processes. These values are of the order previously observed for macromolecular conformation changes [16].

Another class of reducing substances, organic radicals, showed (ref. 17 and Shafferman, A. and Stein, G., unpublished) approx. 100 % efficiency of reduction of native CIII in a reaction the rate of which showed small activation energy but a pre-exponential factor smaller than the possible maximum. This decrease could be due to the requirement of reaction with a specific region, for example that of the heme, or with at least one of two or more equilibrated conformations. These reducing agents showed no evidence of reacting via the protein.

Using H atoms as the reducing agent near neutral pH we found (refs. 17 and 18, and Shafferman, A. and Stein, G., unpublished) that all of these reacted with CIII in a very fast diffusion-limited bimolecular reaction with $k = 1.1 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$. Of the total H atoms which were taken up some 60 % are utilized in reducing CIII to CII, the rest probably forming such peripheral radical adducts on the peptide chain as do not transfer to the heme. Of the 60 % of H atoms which reduce ferri to ferro, about a third do so by reacting directly with the heme region with the observed bimolecular rate constant. Some two-thirds were shown to reduce ferri to ferro in an intramolecular electron equivalent transfer, involving fast primary addition to sites on the protein followed by slower transfer to the heme.

In the present study we investigated the effect on the reduction mechanism of cytochrome *c* by hydrogen atoms of changes in the conformation of CIII caused by going from the native to the acid form by varying the pH. The pH changes lead, inter alia, to an opening of the polypeptide structure [1, 2] and an easier access to the heme. The following results show the kinetic consequences of these changes in protein conformation.

EXPERIMENTAL

Apparatus, procedures and materials. Apparatus, procedures and materials were the same as those employed previously [9, 18] with the following exceptions: Ferri-heme-chloride (approx. 99.5 % pure) purum CHR of Fluka was used without further purification. We used Kremer's procedure [23] for the preparation of ferri-heme solutions, by which the highest polymer content was always less than 8 % of the total ferri-heme monomers at the final pH of 7.0. The ferri-heme was dissolved in 0.01–0.04 M HPO_4^{2-} and $HClO_4$ added (rather than HCl, which interferes in radiation chemistry) to set the final pH. The ferro-heme was obtained by reduction of the ferri-heme with $Na_2S_2O_4$ at pH 7.0.

Difference spectrum between $2 \cdot 10^{-4}$ M CIII and CII at pH 2.0 were measured in a Cary 14 spectrophotometer on stringent deaerated solutions. The reduction (by dithionite) was carried out as described previously [18] at pH 6.8, and deaerated solutions under N_2 (oxygen free) were brought to pH 2.0 by addition of $HClO_4$. Measurements on pulsed solutions at $\lambda > 680$ nm were obtained using R-406 Hamamatsu photomultiplier instead of RCA IP28, at $\lambda < 680$.

RESULTS AND DISCUSSION

(a) *Reduction of ferricytochrome c (CIII) by hydrogen atoms at pH 3.0–3.2*

First we report the extension of our study (refs. 17 and 18, and Shafferman, A. and Stein, G., unpublished) of the reduction of CIII by hydrogen atoms at pH 3.0–3.2 (where the protein configuration and our results were similar to that at neutral pH) to

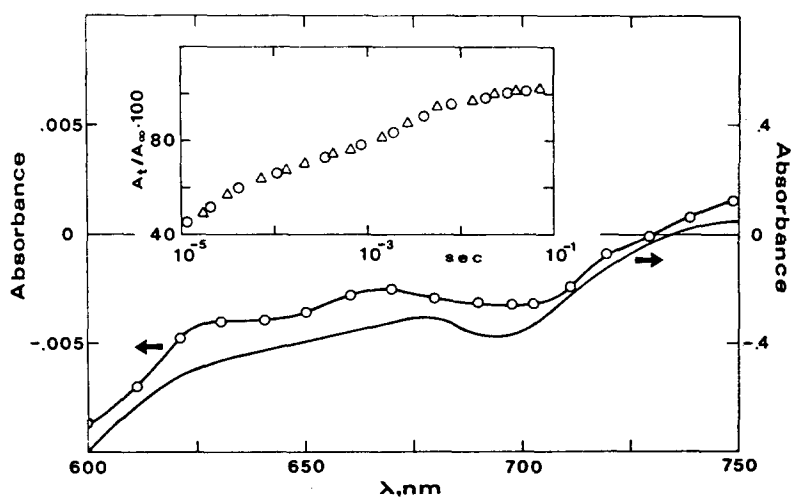

Fig. 1. Difference spectrum between reduced and oxidized cytochrome *c* at pH 3.2. ○—○, difference spectrum between $1 \cdot 10^{-5}$ M CIII in the presence of 0.1 M *tert*-butanol (light path, 12.3 cm) and the same solution 500 ms after a 100 ns pulse of approx. 300 rad; —, difference spectrum (measured in a Cary 14 spectrophotometer) between $1.5 \cdot 10^{-4}$ M CIII at pH 6.8 (light path, 4 cm) before and after complete reduction with dithionite (phosphate buffer, $5 \cdot 10^{-2}$ M). Insert: intramolecular processes. Variation of A_t/A_∞ ($\lambda = 640$ nm) with $\log t$ at pH 3.2, $[CIII]_0 = 3 \cdot 10^{-5}$ M. ○, ≈ 300 rad per pulse; △, ≈ 700 rad per pulse.

spectral changes in the region of 580–750 nm, beyond the previously covered range of 320–580 nm. This extension was required since much of the new information from the reaction of hydrogen atoms with acid, CIII (pH $\approx$ 2.0) in which the clearest results were obtained, was in the spectral region above 600 nm. Fig. 1 shows the difference spectrum from 600 to 750 nm obtained 500 ms after a pulse of 100–300 ns given to a solution of CIII. The results show (refs. 17 and 18, and Shafferman, A. and Stein, G., unpublished) that the product of the reaction between H atoms and CIII is CII produced at this pH with a yield of approx. 55 % of all H atoms that reacted with CIII. This yield accords with the values we obtained previously from the results in the shorter wavelength region.

The results again show in the microseconds to seconds region intramolecular consecutive overlapping processes. Temperature effects (5–35 °C) did not lead to sufficient resolution to separate the processes for detailed kinetic analysis. By a procedure which we have described in detail [18] we could draw some general conclusions about the kinetics involved in the reduction processes. In this procedure we plot the relative absorbance A_t/A_∞ (A_t, absorbance at time t; A_∞, final absorbance) versus the logarithm of time. A typical example is given in Fig. 1 (insert) measured at 640 nm. Such plots at different dose rates and shorter wavelengths were found to be invariant. Changes in the initial CIII concentration produced a variation only on the short time scale of 10^{-6}–10^{-5} s in these plots. From such plots we deduce [18] that all processes ranging from $> 10^{-5}$ s are intramolecular and more specifically that the processes at 10^{-5}–10^{-3} s are mainly intramolecular electron transfer processes, while the intramolecular processes at times $> 10^{-3}$ s are probably due to conformational changes. We reconfirmed in a detailed analysis of the results in the extended spectrum from 600 to 750 nm that at least 70 % of the observed reduction of CIII to CII occurs via these intramolecular processes.

(*b*) *Reduction of CIII by hydrogen atoms at pH 2.0*

The final spectra (Figs. 2 and 3) at pH 2.0 differ from those at pH 3.2 and 6.8 as expected [1]. The agreement between the two curves (Fig. 2) is not as good above 660 nm as below. We do not account for this small discrepancy and the general picture is that at pH 2.0 reduction of CIII by H atoms produces finally CII. However, in contrast to the higher pH range of 3.2–6.7 at pH 2.0 the final product is obtained much faster, in several milliseconds after the pulse, rather than several hundred milliseconds as is the case at pH 3.2 or 6.7. At this acid pH the faster formation of CII is preceded by the formation of an intermediate with a distinctive absorption spectrum. In Fig. 3 the spectrum recorded $5 \cdot 10^{-6}$–$10 \cdot 10^{-6}$ s after the pulse shows a change in absorption produced between 458 and 436 nm with positive sign. The phenomenon is similarly pronounced at 600–750 nm. Here both ferri- and ferrocytochrome *c* absorb weakly so that the kinetics of formation and decay of the absorbing intermediate are clearer. In this region the spectral changes measured $5 \cdot 10^{-6}$–$10 \cdot 10^{-6}$ s after the pulse show that the transient (Fig. 4) has a gradual increase in absorption going from 600 to 750 nm with $\varepsilon_{750} \geqslant 3300\ M^{-1} \cdot cm^{-1}$. This absorbing species decays (insert Fig. 2) and finally produces the absorption of the ferro-enzyme. At higher pH (3.2 or 6.7) there were only minor spectral changes due to intermediate stages of the reduction which did not follow those expected from a direct transition from ferri to the ferro state of the enzyme. At higher pH values the continuous change in the spectrum mea-

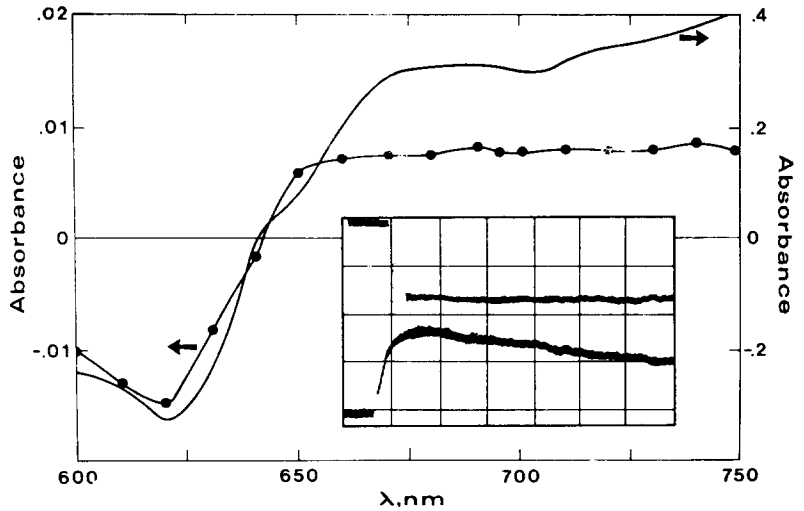

Fig. 2. ● — ●, difference spectrum between $1 \cdot 10^{-5}$ M CIII at pH 2.0 ($HClO_4$) in the presence of 0.1 M *tert*-butanol (light path, 12.3 cm) and the same solution 20 ms after a 100 ns pulse of approx. 400 rad; —, difference spectrum (measured in a Cary 14 spectrometer on stringently deaerated solutions) between CIII and CII ($2 \cdot 10^{-4}$ M) at pH 2.0; light path, 4 cm. Insert: Oscilloscope traces at 630 nm on $1 \cdot 10^{-5}$ M CIII, pH 2.0, 0.1 M *tert*-butanol, approx. 400 rad. Ordinate scale, 2 mV/division; photomultiplier output, 200 mV at $t = 0$. Abscissa scale: upper curve, 20 ms/division; lower curve, 10 μs/division.

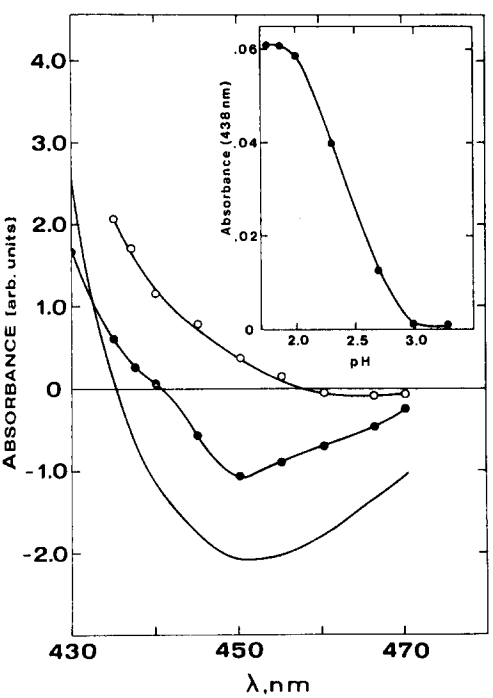

Fig. 3. Difference spectrum due to the intermediate at pH 2.0. –○–, measured (light path, 2 cm) 10 μs after a 100 ns pulse of 400 rad to $1 \cdot 10^{-5}$ M CIII in the presence of 0.1 M *tert*-butanol; ● — ● same conditions but absorption measured 50 ms after pulse showing difference spectrum between CII obtained and original CIII; —, difference spectrum at pH 2.0 between CIII and CII solution obtained by dithionite reduction by procedure as in Fig. 2. Insert: pH dependence of absorbance at 438 nm measured 10 μs after 100 ns pulse of approx. 400 rad.

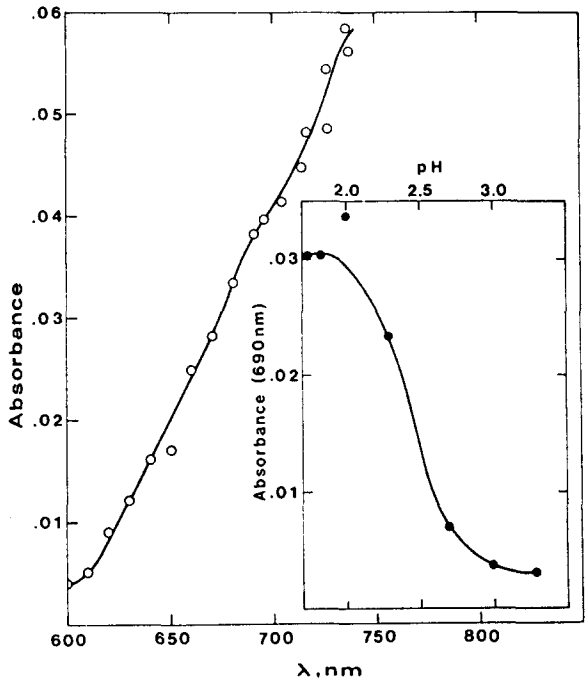

Fig. 4. Difference spectrum due to the intermediate at pH 2.0 measured 10 μs after 100 ns pulse. Other conditions as in Fig. 3, except light path is 12.3 cm. Insert: pH dependence of absorbance at 690 nm.

sured from the shortest times, approx. 500 ns, to about 300 ms, showed some 3–6 % contribution by the new intermediate, the remaining 94–97 % showed changes in absorption at wavelengths as expected from the conversion of the spectrum from that of ferricytochrome directly to ferrocytochrome. The rate of formation of the new absorption produced at 600–750 nm or 440–450 nm was $(2.8 \pm 0.4) \times 10^{10}\ M^{-1} \cdot s^{-1}$, obtained by dividing the observed pseudo first-order rate for the formation of the absorption by the initial CIII concentration. This value is higher by a factor of approx. 3 than that obtained for the reaction of H with CIII at higher pH. Analysis of the kinetics of absorption changes around 550 nm showed that plots of A_t/A_∞ vs. log t were independent of changes in dose. Changes in initial CIII concentration affected the kinetics up to approx. $10 \cdot 10^{-6}$ s only. As discussed before the independence of kinetics of initial CIII concentration and of dose in the time range above 10^{-5} s indicates that the reactions observed there are intramolecular.

Final reduction yields calculated from results at 580, 550, 520 and 460 nm gave 85, 70, 75 and 85 % efficiency, respectively.

Thus, changing the conformation of CIII from the neutral to the acid state affects the reduction mechanism by H atoms, increases the bimolecular rate constant and increases the percentage of reduction.

We attribute the new intermediate observed to the reaction of hydrogen atoms with the porphyrin ligand. This is supported by the following facts: (a) metal porphyrins and radicals of metal porphyrin have an intense absorption band at approx. 600

nm [19, 20]. (b) Acid CIII is known to have an "open" conformation, that is to say that the heme is more exposed to the solution while at higher pH (neutral) only one edge of the porphyrin is exposed to the solvent [1–4]. Thus at low pH the heme is more accessible to the reducing hydrogen radicals. (c) A titration of the absorption (insert, Figs. 3 and 4) either at 430 nm or at 690 nm recorded 10 μs after the pulse yields a pK of 2.4. This value agrees well with the pK [21] for the transition of CIII from type III (cytochrome c at neutral pH) to the type II (acid cytochrome c). (d) NMR studies on the reduction ferri-heme by labeled hydrogen (^{2}H and ^{3}H) show that the ferro-heme produced is labeled by either isotope in the methinic position of the porphyrin, probably due to intermediate addition to the double bond there [22]. All these facts suggest that the transient absorption in question found in the reduction of CIII by hydrogen atoms at pH 2.0 is due to the product of the reaction of the porphyrin ligand with the hydrogen atoms. The reduction of ferri-heme itself by organic radicals or hydrated electrons produces ferro-heme with no detectable intermediates [20], i.e. if an intermediate is formed at all, it is transformed to the stable product with $\tau_{\frac{1}{2}} < 1.4 \cdot 10^{-6}$ s. In the present work we observe an intermediate stable for several microseconds when CIII at pH 2.0 is reduced by H atoms. We have therefore carried out some experiments on the reduction of ferri-heme by H atoms.

(c) Reduction of ferri-heme

We reduced at neutral pH ferri-heme using hydrogen atoms, or, alternatively, ethanol radicals. The results are summarized in Fig. 5. In Fig. 5B we compare the difference spectrum obtained from the reduction of ferri-heme by ethanol radicals with that of chemical reduction with $Na_2S_2O_4$. The product of the reduction by ethanol radicals is ferro-heme obtained in a bimolecular process with $k = 7.7 \cdot 10^8$ $M^{-1} \cdot s^{-1}$, without any detectable formation of intermediates (insert Fig. 5B). Harel and Meyerstein [20] reported that isopropanol radicals reduce ferri-heme at pH 13 in a bimolecular process with $k = 9 \cdot 10^8$ $M^{-1} \cdot s^{-1}$, without any detectable formation of an intermediate.

The difference spectrum of ferri- and ferro-heme had maximum and minimum in absorbance at approx. 560 and approx. 620 nm. The small shift in the spectrum obtained in the pulse radiolytic method relative to the one obtained in the chemical reduction may possibly be due to some ligation by dithionite. In Fig. 5A the results for the reduction of ferri-heme by H atoms at pH $\approx$ 7 are presented. The difference spectrum measured 1 ms after a given pulse is very similar to that shown in Fig. 5B and indicates that at this time the final product, ferro-heme, is present.

In contrast to the organic radical system in the H atoms system one can clearly see an intermediate absorption in the microsecond region. This absorption is different from that expected from the conversion of ferri-heme to ferro. From 500 to 700 nm the intermediate shows an absorption which is always positive in sign. The intermediate absorption in question decays with complex kinetics involving at least two consecutive processes and produces finally the reduced ferro-heme, (insert, Fig. 5A). We conclude that reduction of ferri-heme itself by H atoms produces initially an intermediate which we attribute to an H atom adduct on a methinic position on the porphyrin ligand [22]. From the similarity of the results obtained in the ferri-heme and CII systems it seems that in the latter, reduction by hydrogen atoms occurs also through the porphyrin ligand.

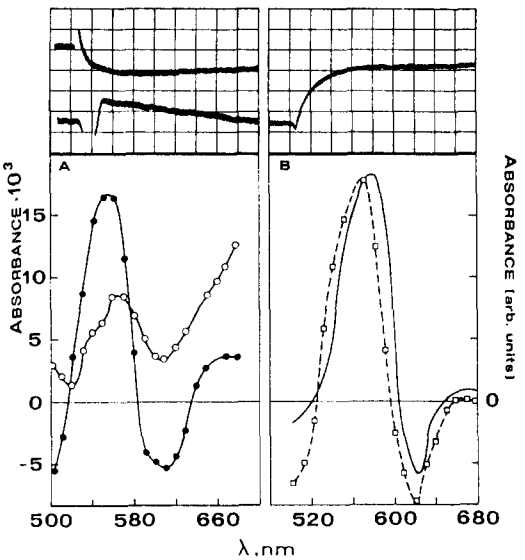

Fig. 5. (A) Difference spectrum after reduction of $2 \cdot 10^{-5}$ M ferri-heme by H atoms at pH 7.0, $5 \cdot 10^{-2}$ M N_2O and 0.2 M *tert*-butanol. Pulse: approx. 1.5 μs, approx. 2700 rad. Difference spectra: ○—○, 5 μs after pulse; ●—●, 1 ms after pulse. Insert: oscilloscope traces at 610 nm. Abscissa scale, lower trace, 5 μs/division, upper trace, 200 μs/division. Ordinate scale, 5 mV/division. Photomultiplier output, $t = 0$, 500 mV. (B) □—□, reduction of $1 \cdot 10^{-5}$ M ferri-heme at pH 7.0 by ethanol radicals (obtained by using 0.1 M ethanol instead of *tert*-butanol); difference spectrum measured 500 μs after a pulse of 1.5 μs, approx. 2700 rad; —, reduction by dithionite of $2 \cdot 10^{-5}$ M ferri-heme; difference spectrum measured on Cary 14 spectrophotometer. Insert: Oscilloscope trace of reduction by ethanol radicals of $1 \cdot 10^{-5}$ M ferri-heme solution measured at 560 nm. Abscissa scale, 100 μs/division; ordinate scale, 5 mV/division. Photomultiplier output at $t = 0$, 200 mV.

In both systems the intermediate, stable for microseconds, shows a gradual increase of absorbance above 600 nm. The extinction coefficients at 640 nm are $\geqslant 350\ M^{-1} \cdot cm^{-1}$ and $\geqslant 800\ M^{-1} \cdot cm^{-1}$ for the heme and the CIII systems, respectively. The wavelength of 640 nm represents an isosbestic point of the ferri and the final ferro state in both systems. The rate of formation of the absorption in question has a lower limit of $3 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ for ferri-heme at pH 7 and $(2.8 \pm 0.3) \times 10^{10}\ M^{-1} \cdot s^{-1}$ for ferricytochrome *c* at pH 2, respectively.

We conclude that at pH 2.0 when CIII is in the more "open" conformation, and the heme more accessible to the solvent, reduction by H atoms proceeds through the heme, and probably through primary addition to the methinic position on the porphyrin ligand.

The question is whether this is the only mechanism which contributes to the electron transfer processes observed at this low pH or whether there is a contribution to these by electron transmission processes from the protein moiety as well. We have calculated the possible relative contributions of each mechanism from the bimolecular rates for the addition of H to CIII at pH 2.0 and 6.7, and from the reduction yields in the two pH ranges, assuming that every H atom which attacks the porphyrin adds an electron equivalent to the iron. The results of these calculations leads us to conclude that the reduction yield of approx. 80 % found at pH 2.0 can be accounted for from

the attack of H atoms only on the porphyrin ligand while at pH 3.0–3.2 and 6.7 the contribution of this mechanism to the reduction of CIII is minor ($< 6\,\%$).

(d) Reduction of CIII by organic radicals at pH 2.0

At neutral pH it was found that organic radicals such as formate, lactate, malate and ethanol reduce CIII in 100 % reduction efficiency; there are no further detectable reactions of the reduced enzyme with these radicals (ref. 17 and Shafferman, A. and Stein, G., unpublished). The reduction is completed during the bimolecular reaction of these radicals with the protein at rates ranging from 10^9 to 10^8 $M^{-1} \cdot s^{-1}$. From the present study on the reduction of ferri-heme by one of these radicals (ethanol) we expect in contrast to the H atom system that reduction of CIII at pH 2.0 will have a similar kinetic behavior to that found at neutral pH (even though the mechanism may differ) in spite of the change in the conformation which exposes the ferriheme at the lower pH. Indeed the kinetics of reduction of CIII at pH 2.0 by ethanol or formate radicals is indistinguishable from that found at higher pH. The only difference lies in the values of the bimolecular rates for the reduction of CIII which is slightly higher at the low pH, namely $7.4 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at pH 6.7 and $9.4 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at pH 2.0 for the reaction of formate radicals (COO^- and COOH); $1.8 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at pH 6.8 and $2.4 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ at pH 2.0 for ethanol radicals. No changes of absorption at $\lambda > 600$ nm other than those expected from the conversion of ferri- to ferro-cytochrome *c* could be detected.

In conclusion we may state that using H atoms as the reducing agent, it was shown that in the closed configuration at neutral pH an appreciable percentage of the reducing equivalent is transferred in ferricytochrome *c* by a mechanism involving primary radical addition to sites on the protein, followed by intramolecular electron equivalent transfer. At acid pH, in the open protein configuration H atoms reach the ferric-heme directly and react there at least partly via addition to the porphyrin. It seems thus that at a physiological pH cytochrome *c* acquires a configuration which enables the polypeptide chain to participate in the electron transfer process, this ability is abolished in the acid conformation. However, this feature of cytochrome *c* is manifested only by the non-physiological reducing agent H atoms.

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