THE REACTION OF FERRIC CYTOCHROME *c* WITH DITHIONITE AT VERY LOW IONIC STRENGTH

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1. Introduction

In a previous communication [1] evidence was presented for a conformation equilibrium of ferric cytochrome *c* in neutral solutions of very low ionic strength. Further support for this hypothesis is provided by the salt dependence kinetic of the reduction of cytochrome *c* with dithionite in neutral solutions described below.

2. Materials and methods

Horse heart cytochrome *c* (Sigma Chem. Co. Type VI) was percolated through Bio-Gel P-4 columns equilibrated with Tris–cacodylate buffer 0.002 M, pH 7.2. Dithionite solutions were prepared by adding solid sodium dithionite (Merck) to buffers that were previously deaerated by a stream of argon during 0.5 h and kept under argon. Other reagents were of analytical grade.

All kinetic experiments were done with a stopped-flow attachment in an Aminco-Chance dual wavelength spectrophotometer at (416–430 nm).

3. Results

Figure 1 shows kinetic measurements of the reduction of 5×10^{-6} M ferricytochrome *c* by 2×10^{-3} M dithionite, at pH 6.7, in Tris–cacodylate buffer 0.002 M. Without added salt (for this solution the contribution of protein, buffer and reductant gives ionic strength, *I* 0.008), the reduction was

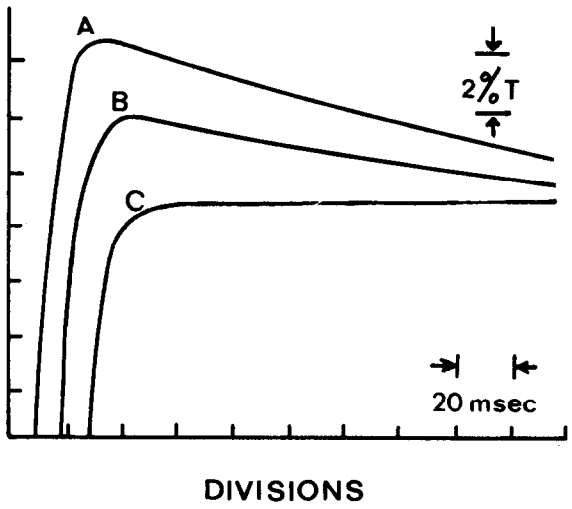

Fig.1. Reduction of 5×10^{-6} M ferricytochrome *c* with 2×10^{-3} M dithionite, at pH 6.7, Tris–cacodylate buffer 0.002 M. (A) Without added salt. (B) With NaCl 0.1 N. (C) With NaCl 0.2 N. Kinetics were followed in a double beam spectrophotometer at 416–430 nm, 25°C. For convenience, only the second phase and the end of the first phase are shown.

clearly biphasic. When the concentration of NaCl was increased, up to *I* 0.208 (fig.1), the second phase progressively disappeared until monophasic kinetics were observed instead. Essentially identical results were obtained when the ionic strength was increased by using more concentrated buffer, or by adding $MgCl_2$, $MgSO_4$ or Na_2SO_4.

The results of the kinetic measurements at different dithionite and salt concentrations are summarized in

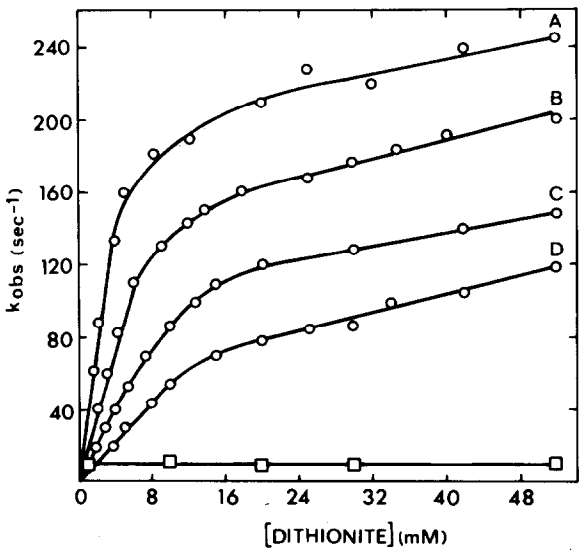

Fig.2. Second order plot for the reduction of 5×10^{-6} M ferricytochrome *c* with dithionite at 25°C, 416–430 nm in 0.002 M Tris–cacodylate buffer, pH 6.7. (○) For the rapid (initial) phase, at various NaCl concentrations: (A) 0-NaCl, (B) 0.1 N NaCl, (C) 0.4 N NaCl, (D) 1.0 N NaCl. (□) for the slow phase, at NaCl concentrations: 0, 0.05, 0.1 M.

fig. 2. At very low I, it is apparent that the fast phase of the reaction features different mechanisms at high and low dithionite concentrations, similarly to the behaviour at very high ionic strength (1 N NaCl) [2]. Creutz and Sutin [2] suggested two different bimolecular pathways for the fast reduction by dithionite: one, a direct remote attack; the other, an adjacent pathway requiring the opening of the heme crevice. We have evaluated the limiting rate constants for the opening of the heme crevice as a function of salt concentration (table 1).

Table 1

NaCl (N)	k^a (s^{-1})
0	150
0.1	130
0.2	110
0.4	90
1.0	45

[a] Limiting rates, k, for the fast phase of the reduction of cytochrome *c* with dithionite, calculated from fig.2

The slow phase, following the reduction, takes place in the time range of milliseconds ($t_{1/2}$ 70 ms) and its rate is independent of protein, dithionite or salt concentration (in the limits of salt concentration where it is possible to measure) (fig.2). The observed specific rate for the slow phase is 10 s^{-1} for 1–50 mM dithionite (fig.2). The amplitudes of the absorbance changes of the slow phase were measured in the Soret region and are plotted in fig.3. The difference spectrum between the stable form of reduced cytochrome *c* and the reduced intermediate shows only changes in the absorbance but no spectral shifts. This difference spectrum is very similar to that observed for the same reaction at alkaline pH, and moderate ionic strength [3,4]. However, the absorption changes observed for

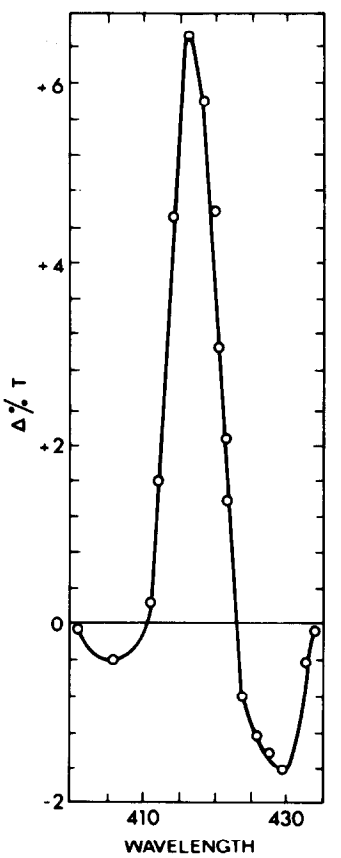

Fig.3. Difference spectra between the reaction product and intermediate after reduction of 5×10^{-6} M ferricytochrome *c* with 10 mM dithionite in 0.002 M Tris–cacodylate buffer, pH 6.7.

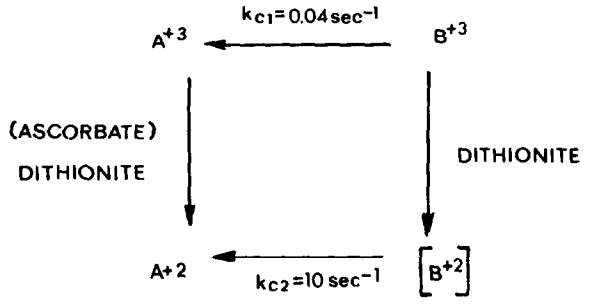

Fig.4. Suggested scheme for the reduction of cytochrome *c* at pH 7, $I \rightarrow 0$, with dithionite and with ascorbate.

neutral, low ionic strength solutions, were very small, making it impossible to determine them with sufficient precision in the visible region.

4. Discussion

The biphasic kinetics of the reduction of cytochrome *c* by ascorbate at neutral pH was explained [1] by the hypothesis that in the absence of salts, at $I \rightarrow 0$, ferric cytochrome *c* is present in at least two different conformations: one, A^{+3}, that reacts rapidly with ascorbate; and another, B^{+3}, that reacts very slowly, or not at all (fig.4). The rapidly reacting form, A^{+3}, accounts for 80–90% of the total protein. The slow phase of the reaction corresponds to the conversion of the second form, B^{+3}, into the first, A^{+3}. Since the observed rate constant for the transition $B^{+3} \rightarrow A^{+3}$, calculated from the slow phase of ascorbate reduction, is 0.04 s^{-1} [1], the rate constant for $A^{+3} \rightarrow B^{+3}$ is approx. 0.01 s^{-1}. The addition of salts shifts the conformation equilibrium towards the first reducible, A^{+3}, form, and the reduction becomes monophasic.

When dithionite is used for the reduction of ferric cytochrome *c*, a reduced intermediate, $[B^{+2}]$ (fig.4), is formed (fig.1, fig.3); this was not observed during the reduction by ascorbate. This difference results from the much faster kinetics observed for dithionite. It is obvious that the reduction of cytochrome *c* by dithionite proceeding in the first phase is much faster than any possible interconversion between the oxidized conformers A^{+3} and B^{+3}: in the absence of salt and at the lowest dithionite concentration used (1 mM), k_{obs} for the fast phase is 70 s^{-1} (fig.2) while the rate constant of the transition $B^{+3} \rightarrow A^{+3}$ is 0.04 s^{-1} [1], and in the opposite direction ($A^{+3} \rightarrow B^{+3}$) about 0.01 s^{-1}. The redox potential of the B^{+3} conformer is unknown and we can only assume that dithionite, unlike ascorbate, is capable of reducing both conformers (A^{+3}, B^{+3}) at fast rates that cannot be resolved, resulting in the single fast phase observed (fig.1). Consequently, that part of the reduction which proceeds through the B^{+3} conformer creates an unstable intermediate $[B^{+2}]$ which converts to the final reduced stable state A^{+2} (fig.1, fig.4), in a monomolecular transition with a specific rate k_{c2} 10 s^{-1}.

The data for the reduction of ferricytochrome *c* by dithionite is in partial agreement with the results of pulse radiolysis [5,6] where ferricytochrome *c* is reduced by hydrated electrons. The major difference concerns observations near neutrality where Pecht and Faraggi [5] reported slow secondary spectral changes while Land and Swallow [6] saw none. Lambeth et al. [3], in agreement with Land and Swallow [6], found the reduction of cytochrome *c* by dithionite to be biphasic only in the alkaline region, and tried to explain Pecht's data by poor control of pH in unbuffered solutions [3]. Pecht and Faraggi [5] found the specific rate for the slow transition to be 8.5 s^{-1}, very similar to the specific rate (k_{c2} 10 s^{-1}) for the slow interconversion of the unstable $[B^{+2}]$ conformer found by us in neutral, very low ionic strength buffered solution (Tris–cacodylate 0.002 M); so that we attribute Pecht's findings to the low ionic strength of the medium rather than to improper pH caused by unbuffered solutions. Nevertheless it is impossible to conclude that the transient reduced forms in neutral pH are all the same when dithionite or aquated electrons are used as reductants, as we, unlike Pecht [5] do not observe any spectral shift (fig.3) but only slight changes in absorbance.

It is quite obvious that the reduction studied at very high ionic strength (1 N NaCl) at neutral pH [2] was monophasic, because such conditions permit the existence of only one ferric cytochrome *c* conformer, A^{+3}. However, the first fast phase of the reduction is dependent on dithionite concentrations, showing saturation in rate at high concentrations of reductant at very low ionic strength as well as at higher NaCl concentrations (fig.2). It is only the extent of saturation that differs at different salt concentrations. Analyzing the data for the first phase of reduction

according to the mechanism described by Creutz and Sutin [2], we conclude that the limiting rates for the opening of the heme crevice (table 1) are dependent on salt concentration so that absence of salt facilitates the opening of the heme crevice (k 150 s^{-1}) while high concentration of NaCl (1 N NaCl) may impose a more rigid structure and a consequent lower rate of crevice opening (k 45 s^{-1}), (table 1).

Acknowledgements

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References

[1] Goldkorn, T. and Schejter, A. (1977) FEBS Lett. 75, 44–46.
[2] Creutz, C. and Sutin, N. (1973) Proc. Natl. Acad. Sci. USA 70, 1701–1703.
[3] Lambeth, D. O., Campbell, K. L., Zand, R. and Palmer, G. (1973) J. Biol. Chem. 248, 8130–8136.
[4] Schejter, A., Aviram, I., Margalit, R. and Goldkorn, T. (1975) Ann. NY Acad. Sci. 244, 51–59.
[5] Pecht, I. and Faraggi, M. (1972) Proc. Natl. Acad. Sci. USA 69, 902–906.
[6] Land, E. J. and Swallow, A. J. (1971) Arch. Biochem. Biophys. 145, 365–373.