THE ASCORBATE REDUCTION OF DENATURED FERRICYTOCHROME c*

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Summary: The reduction of ferricytochrome c by ascorbate in solutions of the denaturants, 1-propanol and urea, proceeds under conditions where the 695 nm absorbance band is completely quenched, thus indicating that the heme iron-methionine 80 bond is not an essential requisite for the reduction of the iron of ferricytochrome c by ascorbate. The implications of these results for the elucidation of the mechanism of action of cytochrome c are discussed. The denaturant induced autoxidation of ferrocytochrome c was used in monitoring the reduction indirectly with an oxygen electrode.

Elucidation of the mechanism of action of cytochrome c involves the determination of the pathways whereby electrons enter and leave the heme iron. Many studies of the oxidation and reduction of cytochrome c have been performed with this object in view (1), and in particular the reduction of ferricytochrome c by ascorbate has been well investigated (2,3); Wilson and Greenwood (4) have reported that such a reduction can only proceed when the heme iron methionine 80 bond is intact, as evidenced by the presence of the 695 nm absorbance band. Reductions of this type are usually monitored spectrophotometrically at 550 nm. In solutions of denaturants such measurements are suspect for two reasons: at high denaturant concentrations the ferrocytochrome c becomes autoxidisable thus masking any reduction which may occur (5) and, under certain conditions the 550 nm band of ferrocytochrome c can disappear although the heme iron remains in the reduced state (6). We report here our studies on the

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reduction of cytochrome c by ascorbate in the presence of denaturants which completely quench the 695 nm band, using a system which overcomes the problems of the spectrophotometric assay.

Materials and Methods

Horse heart cytochrome c (Miles-Seravac, 95% pure, Grade 1, 0.425%Fe) was oxidised with potassium ferricyanide and purified by passage through a Sephadex G-25 column. Aqueous solutions of ascorbic acid (Merk, Analytical Reagent) were prepared immediately before use. Buffered alcoholic and urea solutions were prepared as previously described (5) and were corrected for shrinkage and pH shifts. Water was distilled and deionized.

Oxygen consumption was determined using a Yellow Springs Instrument Clark type oxygen electrode in a thermostatted water bath ($\pm$ 0.02°). Reaction solutions were made up as follows: the buffered solutions of denaturant and ascorbate (0.01M) were mixed and thermally equilibrated in the presence of the oxygen electrode. After a stable base line was obtained the reaction was initiated by the addition of an aqueous solution of ferricytochrome c (10 μM) and the rate of change of oxygen content of the solution was recorded. Denaturant induced absorbance changes at 695 nm were determined with a Beckman DBGT spectrophotometer. The residual absorbance of the 530 nm absorbance band was subtracted from the reported readings of the 695 nm absorbance.

Results and Discussion

The rates of oxygen consumption of solutions of ferricytochrome c and ascorbate incorporating varying concentrations of

1-propanol or urea are compared with the absorbance at 695 nm of ferricytochrome c in solutions of the same concentrations of denaturants (Figures 1 and 2). In the presence of both

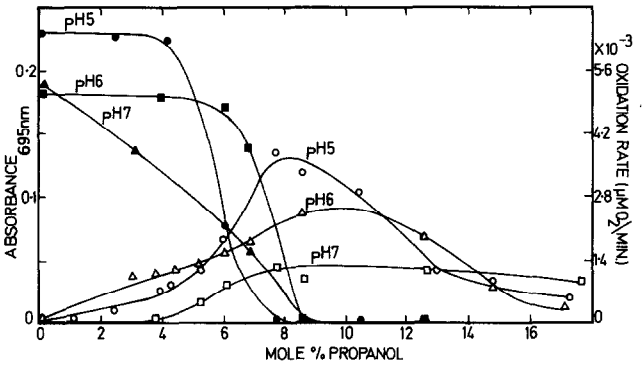

Fig. 1. The effect of 1-propanol on the rate of reduction by ascorbate and the 695 nm absorbance of ferricytochrome c. O, □, Δ, rates of reduction measured indirectly using oxygen consumption rates; ●, ■, ▲, 659 nm absorbance. Cytochrome c 10 μM, 0.1 M acetate buffer, 25°.

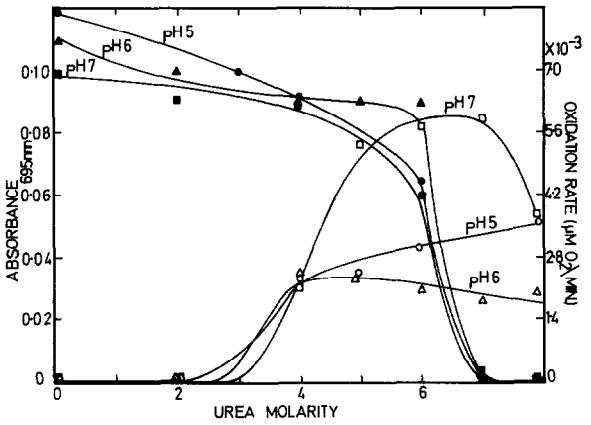

Fig. 2. The effect of urea on the rate of reduction by ascorbate and the 695 nm absorbance of ferricytochrome c. Other details as in Fig. 1.

denaturants at pH 5, 6, and 7 the consumption of oxygen proceeds at concentrations of denaturant far greater than those which produce complete quenching of the 695 nm absorbance of the protein. When the reaction mixtures were constituted without either ascorbate or ferricytochrome c or both no oxygen consumption was noted except at high concentrations of urea where the rate of autoxidation of ascorbate became relatively fast and was subtracted from the rates in the presence of ferricytochrome c.

In view of our earlier findings (5), that ferrocytochrome c becomes susceptible to direct oxidation by oxygen (autoxidizable) in solutions of propanol and urea, it is clear that the oxygen consumption observed in these experiments must result from the autoxidation of ferrocytochrome c. As a consequence the ferricytochrome c must be reduced by ascorbate to yield ferrocytochrome c even at high concentrations of denaturants where the 695 nm band of the ferricytochrome c is completely quenched (Figures 1 and 2).

The slow oxidation rates at low denaturant concentrations (Figures 1 and 2) result from slow or zero autoxidation rates (5) and not from a failure to reduce. The decline in oxidation rates at very high denaturant concentrations probably results from a decreased rate of reduction which may be a consequence of the more extensive denaturation of the protein (6).

There has been much speculation concerning the pathway of electrons into the heme iron of ferricytochrome c. The two most likely routes are either through the conjugated heme system which has been shown to have an edge exposed to the surrounding solvent (7), or through the protein itself via the methionine 80 sulphur atom liganded to the iron. The results

of Wilson and Greenwood (4) imply the latter pathway is the more likely *in vitro* with ascorbate as reductant since cleavage of the methionine heme iron bond appeared to prevent the reduction. Our results however indicate that cleavage of this bond, as evidenced by the quenching of the 695 nm band absorbance (8),does not prevent reduction, and demonstrate that the presence of the methionine sulphur is not essential for the passage of electrons from ascorbate into the heme iron.

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