Electron Transfer between Soluble and Immobilized Mammalian Cytochrome *c*

EQUILIBRIUM AND KINETIC STUDIES ON IMMOBILIZED CYTOCHROME *c*

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Horse heart cytochrome *c* was covalently bound to Sepharose 4B and its redox properties were measured under various experimental conditions. The equilibrium constant for the electron exchange between the oxidized and the reduced form of cytochrome *c* when one of the two forms was in the semi-solid state and the other one in solution was close to 1. Matrix-bound ferrocytochrome *c* is very stable to autoxidation and is not oxidized by O₂ even in the presence of mammalian cytochrome oxidase. Oxidation occurs if catalytic amounts of soluble cytochrome *c* are added to the reaction mixture. The rate of oxidation of matrix-bound ferrocytochrome *c* in the presence of cytochrome oxidase and catalytic amounts of soluble cytochrome *c* may be correlated with the rate of electron transfer between soluble and matrix-bound cytochrome *c*. This rate is more than two orders of magnitude lower than that reported for the homonuclear (between identical species) electron transfer in solution.

A systematic investigation has been undertaken for several years in this laboratory to explore the properties of various haem proteins covalently bound to solid matrices (Giacometti *et al.*, 1972; Colosimo *et al.*, 1973). Special interest in the subject is justified by the consideration that the complex interrelationships of proteins of the respiratory chain with mitochondrial membranes *in vivo* could reflect a different functional behaviour from that observed in solution. The present paper reports studies on the equilibria and kinetics of electron-transfer reactions of immobilized horse heart cytochrome *c*.

Materials and Methods

Cytochrome *c* from horse heart was obtained from Boehringer Mannheim G.m.b.H. (Mannheim, Germany) and used without further purification. Cytochrome *c* oxidase, isolated by the method of Yonetani (1960) from ox heart mitochondrial particles [the purified material being dissolved in 0.1 M-potassium phosphate buffer, pH 7.4, containing 1% (v/v) Tween 80], was kindly given by Dr. C. Greenwood (School of Biological Sciences, University of East Anglia, Norwich, U.K.). Protein concentrations were measured spectrophotometrically by using the following molar extinction coefficients: reduced horse cytochrome *c*, $\epsilon_{550} = 27\,600 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$

(Schejter *et al.*, 1963), and reduced cytochrome *c* oxidase $\epsilon_{605} = 21\,000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Yonetani, 1961).

Sepharose 4B was from Pharmacia Fine Chemicals (Uppsala, Sweden), CNBr from Fluka A.G. (Buchs S.G., Switzerland), and Tween 80 from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other reagents were of analytical grade and were used without further purification.

Preparation of Sepharose 4B–cytochrome c covalent complex

Chemical coupling between oxidized cytochrome *c* and Sepharose 4B was achieved by using CNBr and following the general method of Axen & Ernback (1970) with only minor modifications.

In a standard preparation 50 ml of cytochrome *c* solution (8 mg/ml) was added to 50 ml of CNBr-activated Sepharose 4B, and the suspension at pH 9 was stirred overnight at 4°C; under these conditions the yield of immobilized protein was almost quantitative.

To make the matrix–protein complex obtained under such conditions suitable for spectrophotometric measurements, it was ‘diluted’ by mixing with Sepharose 4B treated in the same way as above, but in which the protein–matrix ratio during the coupling process was lower by a factor of 20.

Equilibrium and kinetic experiments

Two types of experiments were performed, namely (a) batch experiments and (b) flow experiments. Measurements of the former type were performed by mixing at room temperature (21°C) about 10 ml of the matrix-protein complex suspension in the reduced or oxidized form with the same or different molar amount of soluble protein in oxidized or reduced form respectively. After 20 min of slow magnetic stirring, the soluble cytochrome *c* was separated by simple centrifugation (5000*g* for 15 min), and its spectrum recorded.

The bottom attachment of a flow cuvette was covered by a thin piece of filter paper to support the gel beds, and then connected to a peristaltic pump. The upper attachment was connected to the vessel containing the suspension of the insoluble cytochrome *c*. By action of the peristaltic pump the cuvette was carefully filled up and settled into the cell compartment of the spectrophotometer. All the soluble reagents (i.e. the reductant $\text{Na}_2\text{S}_2\text{O}_4$, the soluble cytochrome *c* and the cytochrome oxidase, all in 0.2 M-potassium phosphate buffer, pH 7.4), as well as the equilibrating buffer (same as above), were then passed through the insoluble cytochrome *c* contained in the cuvette simply by connecting the upper attachment to the various vessels, and the changes in absorbance

recorded as a function of time. Flow rates were in all cases 40 ml/h.

To test for possible spurious effects caused by the gel beds, soluble cytochrome *c* was oxidized by cytochrome oxidase in the presence and absence of suspended gel particles, as well as of 1% Tween 80. In all cases the differences between the observed initial velocities (V_{in}) were in the range of experimental errors.

Results

Spectroscopy

Fig. 1 shows the spectra in the visible region of the reduced and oxidized derivatives of matrix-bound cytochrome *c*. It was found that the small absorption band at 695 nm, which is characteristically observed in native cytochrome *c* in the ferric form (Lemberg & Barrett, 1973), is present also in the matrix-bound enzyme. In addition it was found that this band reversibly disappears on increase in temperature to around 70°C. These observations taken together point to the integrity of the active site of the molecule after binding to the resin, at least as far as the haem ligands are concerned (Schejter & George, 1964).

Equilibrium

Equilibrium experiments were carried out by the

Fig. 1. Time-dependence of oxidation of insoluble cytochrome *c* followed spectrophotometrically at 550 nm

A solution of 99 nm-cytochrome oxidase and 11 μM -cytochrome *c* was pumped at a rate of 40 ml/h into the flow cuvette (light-path 2 mm; total volume 0.7 ml). (a), (b) and (c) are the spectra in the visible region of (respectively) insoluble cytochrome *c* reduced by $\text{Na}_2\text{S}_2\text{O}_4$, the same as in (a) after the passage of 3 ml of 0.2 M-potassium phosphate buffer, pH 7.1, and the same as in (b) after the passage of the solution specified above. The recording chart speed was 2 in/min, and 1 in corresponds to three squares. The temperature was 27°C.

batch method, mixing different molecular ratios of matrix-bound and soluble cytochrome *c* in different oxidation states. The results, shown in Table 1, indicate that the equilibrium constant between soluble and bound cytochrome *c* is very near to unity, and thus the redox potential of cytochrome *c* is unchanged on binding to the solid matrix.

To avoid errors due to autooxidation of soluble reduced cytochrome *c*, blank experiments were run in parallel. However, it was observed that bound cytochrome *c* remains stable in the reduced form for a long time (10–15% autooxidized after 8 h).

Kinetics

The reduced form of matrix-bound cytochrome *c* is not autooxidizable, but, even more significantly, it

Table 1. Equilibrium constant for the reactions between soluble and insoluble cytochrome *c*

Row I refers to experiments in which the fully oxidized and soluble cytochrome *c* was mixed with the fully reduced and insoluble derivative; row II refers to the opposite situation. The numbers in parentheses are molar ratios soluble/insoluble cytochrome *c* used in each case. All the experiments were performed in 0.2M-potassium phosphate buffer, pH 7.1, and the temperature was 25°C.

I	1.00 (0.5)	1.17 (1.0)	1.25 (2.0)
II	0.78 (0.6)	1.00 (1.0)	0.70 (1.0)

Fig. 2. Dependence on cytochrome oxidase concentration of the insoluble cytochrome *c* oxidation in the presence of soluble cytochrome *c*

The insoluble cytochrome *c* concentration was 170 μ M and that of soluble cytochrome *c* was 11 μ M in 0.2M-potassium phosphate buffer, pH 7.1. The temperature was 27°C. ●, Experiments carried out under continuous flow of solution through the cuvette; ○, the peristaltic pump was stopped at the very beginning of the reaction.

Fig. 3. Dependence on soluble cytochrome *c* (Cyt_s) concentration of the insoluble cytochrome *c* oxidation catalysed by cytochrome oxidase

The insoluble cytochrome *c* concentration was 170 μ M and that of cytochrome oxidase 940 nM. All other conditions are as described for Fig. 2.

was found that it is not quickly oxidized by O_2 even in the presence of cytochrome oxidase. For example, in one experiment in which total immobilized cytochrome *c* was 23 nmol and total cytochrome oxidase was 1.5 nmol, after about $\frac{1}{2}$ h only 22% of the cytochrome *c* was oxidized.

At present it is difficult to attribute unequivocally this behaviour either to (i) steric effects due to restrictions in the degree of freedom of cytochrome *c* or to (ii) a specific modification of lysine-13 and/or lysine-14, which are known to be specific basic residues required for the interaction with the oxidase (Dickerson *et al.*, 1971; Wada & Okunuki, 1968). However, as expected from the equilibrium results, oxidation of matrix-bound cytochrome *c* occurs in the presence of cytochrome oxidase if catalytic amounts of soluble cytochrome *c* are added to the suspension (Fig. 1).

Initial oxidation rates of matrix-bound cytochrome *c* as a function of the concentrations of cytochrome oxidase and soluble cytochrome *c* are reported in Figs. 2 and 3 respectively. Fig. 2 shows that the initial velocity is independent of cytochrome oxidase concentration above 0.5 μ M even if the flux of reagents, namely cytochrome oxidase and oxidized cytochrome *c*, is stopped as soon as the cuvette is filled up, as monitored by the oxidation of insoluble cytochrome *c*. If the flux is not stopped the oxidation is determined mostly by direct electron exchange with the soluble oxidized cytochrome *c*, and therefore at low cytochrome concentrations (e.g. below 0.5 μ M) the initial rate constant is larger.

More striking is the trend of the graph shown in Fig. 3. In fact, at the chosen, fully saturating, cyto-

chrome oxidase concentration, a linear relationship between oxidation rates and soluble cytochrome concentrations should be expected; on the contrary, however, the curve approaches an asymptote very rapidly.

Oxidation by an excess of ferricyanide and reduction by $\text{Na}_2\text{S}_2\text{O}_4$ of matrix-bound cytochrome *c* carried out under the same conditions showed initial rates higher by a factor of about 10 than those measured and reported in Figs. 2 and 3. This excludes the possibility that the rate-limiting process observed in Fig. 3 is determined by the maximum flux velocity through the cuvette used in our experimental set-up.

From the linear portion of the curve in Fig. 3 a value of $1.25 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ is obtained for the second-order kinetic constant of the homonuclear electron-transfer reaction between the two cytochromes, namely the soluble and the bound. Kowalsky (1965) reported for the same process studied in solution a value of $5(\pm 3) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, that is more than two orders of magnitude higher.

Discussion

Immobilization of cytochrome *c* on dextran gel by the CNBr procedure is not associated with drastic changes in the overall properties of the protein. In fact, matrix-bound cytochrome *c* differs from the native molecule less than other soluble, but chemically modified, forms such as acetylated and succinylated cytochrome *c*. Thus the spectroscopic features, the reversible thermal transition of the 695 nm band and the equilibrium measurements indicate a substantial integrity of bound cytochrome. Wada & Okunuki (1968) calculated a value for E_0 of 190 mV for the completely acetylated form (22 mol of acetyl group/mol of cytochrome *c*), taking the redox potential of the native molecule as 255 mV at pH 7. In the same paper they also reported that acetylated cytochrome *c* is quickly oxidized in the presence of O_2 and has CO-binding capacity; neither of these features were observed in the matrix-bound molecule.

On the other hand both acetylated and matrix-bound cytochrome *c* share the property of being unable to transfer electrons rapidly to cytochrome oxidase. The lack of direct electron transfer between bound cytochrome *c* and cytochrome oxidase may be due (i) to chemical changes involving lysine-13 and lysine-14, which are known to be essential for the interaction with the oxidase (Dickerson *et al.*, 1971), and/or (ii) to steric-hindrance effects offered by the matrix to the interaction between the two proteins.

Major difficulties arise in the interpretation of the kinetic data on electron transfer between bound and soluble cytochrome *c*, because of the results shown in Fig. 3. The kinetics of electron transfer between the two cytochromes may be described by:

$$-\frac{d[\text{cyt}_b^{2+}]}{dt} = k[\text{cyt}_b^{2+}][\text{cyt}_s^{3+}] \quad (1)$$

where cyt_b = matrix-bound cytochrome *c* and cyt_s = soluble cytochrome *c*. Under the experimental conditions used, because of the presence of sufficient amounts of cytochrome oxidase in the reaction mixture, it can be assumed that the soluble cytochrome *c* will be always present in the oxidized form. Thus eqn. (1) becomes:

$$-\frac{d \ln[\text{cyt}_b^{2+}]}{dt} = k' \quad (2)$$

where $k' = k[\text{cyt}_s^{3+}]$. As shown by the results given in Fig. 3, however, the system does not conform to this simple prediction, since the measured rate is linear in $[\text{cyt}_s^{3+}]$ only in the lower concentration range and tends towards a limit at higher concentrations. This effect, however, cannot be solely due to immobilization, as shown by the fact that a similar kinetic pattern has been observed in experiments of the same type with the reagents all in solution, i.e. both in the oxidation of acetylated cytochrome *c* in the presence of native cytochrome *c* and cytochrome oxidase (A. Colosimo, unpublished work) and in the oxidation of *Pseudomonas* cytochrome c_{551} in the presence of horse heart cytochrome *c* and mammalian cytochrome oxidase (Greenwood *et al.*, 1971).

The very small value of the second-order rate constant for electron exchange between soluble and bound cytochrome *c* ($1.25 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) deserves comment, especially considering that the redox potential of bound cytochrome *c* is unchanged. This rate should be compared with (a) that for the homonuclear transfer in soluble cytochrome *c*, for which a value of $5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ has been reported (Kowalsky, 1965), and (b) that for the reaction between acetylated and normal cytochrome *c* ($1.1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; A. Colosimo, unpublished work). The latter result may indicate that chemical changes of the amino groups are not responsible for the drastic decrease in electron-transfer rate if charge effects can be neglected. Although at this stage no clear-cut explanation for the large decrease in electron transfer between bound and soluble cytochrome *c* can be offered, the most likely interpretation may be foreseen in restrictions to the diffusion rate of soluble cytochrome *c* through the matrix. No attempt has yet been made to account for this effect quantitatively.

It may be pertinent to recall that effects on rates of reaction have been often observed with enzymes covalently bound to matrices and have also been tentatively explained in the framework of models which take into account diffusional effects (for a review see Katchalsky *et al.*, 1971).

References

- Axen, R. & Ernback, S. (1970) *Eur. J. Biochem.* **18**, 351–360
- Colosimo, A., Stefanini, S., Brunori, M. & Antonini, E. (1973) *Biochim. Biophys. Acta* **328**, 74–80
- Dickerson, R., Takano, T., Eisenberg, D., Kallai, O., Samson, L., Cooper, A. & Margoliash, E. (1971) *J. Biol. Chem.* **246**, 1511–1535
- Giacometti, G. M., Colosimo, A., Stefanini, S., Brunori, M. & Antonini, E. (1972) *Biochim. Biophys. Acta* **285**, 320–325
- Greenwood, C., Finazzi-Agrò, A., Guerrieri, P., Avigliano, L., Mondovi, B. & Antonini, E. (1971) *Eur. J. Biochem.* **23**, 321–327
- Katchalsky, E., Silman, I. & Goldman, R. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol.* **34**, 445–536
- Kowalsky, A. (1965) *Biochemistry* **4**, 2382–2388
- Lemberg, R. & Barrett, J. (1973) *Cytochromes*, p. 172, Academic Press, London and New York
- Schejter, A. & George, P. (1964) *Biochemistry* **3**, 1045–1049
- Schejter, A., George, P., Glauser, S. C. & Margoliash, E. (1963) *Biochim. Biophys. Acta* **73**, 641–643
- Wada, K. & Okunuki, K. (1968) *J. Biochem. (Tokyo)* **64**, 667–681
- Yonetani, T. (1960) *J. Biol. Chem.* **235**, 845–852
- Yonetani, T. (1961) *J. Biol. Chem.* **236**, 1680–1688