Kinetics of Reaction of [Nitrotyrosyl]Cytochrome *c* with Ligands

By AGUINALDO JOSÉ DO NASCIMENTO* and KAZUKO HISHIDA DO NASCIMENTO
School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

(Received 17 February 1976)

The reaction of [nitrotyrosyl]cytochrome *c* with ligands was studied by stopped-flow techniques. At pH 7.0 the reaction with imidazole shows two distinct phases, one fast phase being concentration-dependent and a slow phase being concentration-independent. The results are consistent with the existence of two forms of [nitrotyrosyl]cytochrome *c* in solutions [Schejter *et al.* (1970) *Biochemistry* 9, 5118–5122]; form I, the smaller fraction, seems to be responsible for the slow first-order process.

Very few kinetic data exist for the ligand-binding reaction of cytochrome *c*. Indeed, the native molecule has a biological role in oxidation–reduction reactions and probably does not bind ligands under conditions *in vivo*.

Sutin & Yandell (1972) analysed the reaction of native cytochrome *c* with various ligands and found a rate-limiting constant of 60 s⁻¹ for the rupture of the iron–sulphur bond involving methionine-80. They postulated a limiting S_N1 mechanism (monomolecular nucleophilic substitution) in which an intermediate species of highly reactive open-crevice ferricytochrome *c* is formed in the reaction. This activated species could be a stable penta-co-ordinated iron(III) of ferricytochrome *c* or one in which the methionine-80 sulphur atom is replaced by a water molecule as the iron ligand (Sutin & Yandell, 1972).

In previous papers we analysed some physicochemical properties of [nitrotyrosyl]cytochrome *c* (do Nascimento, 1976) and the reactivity of the nitrated protein with ascorbate (do Nascimento & do Nascimento, 1976).

The work reported here was undertaken in an attempt to acquire more information about the reactivity and structural features of [nitrotyrosyl]cytochrome *c* and to compare them with the native molecule.

Materials and Methods

Native cytochrome *c* (type III) and imidazole were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and were used without further purification. Iminazole was from BDH, Poole, Dorset, U.K. The concentration of native cytochrome *c* solutions was measured by using a millimolar extinction coefficient of 27.7 litre·mmol⁻¹·cm⁻¹ at 550 nm for the reduced protein (Margoliash, 1954).

* Present address: Universidade Federal do Paraná, Departamento de Bioquímica, Travessa Alfredo Buffren S/N, Cx. Postal 939, 80.000 Curitiba PR, Brazil.

[Nitrotyrosyl]cytochrome *c* was prepared and assayed as described by do Nascimento (1976).

Dithionite was from Hardman and Holden, Manchester, U.K.

Stopped-flow measurements were carried out by using an apparatus identical with that described by Gibson & Milnes (1964). All static spectrophotometric measurements were made with a Pye Unicam SP. 700A recording spectrophotometer.

The kinetic data were treated according to the mechanism proposed by Sutin & Yandell (1972).

where X represents the ligand added, ferricytochrome *c** the intermediate cytochrome *c* species, and ferricytochrome *c*-X the complex formed between the cytochrome *c* and ligand X. This mechanism yields eq. (3) for the observed pseudo-first-order rate constant, assuming that the steady-state approximation of $k_{-1} \geq (k_{+1} + k_{+2})$ is valid.

$$\frac{1}{\alpha k_{\text{obs}}} = \frac{1}{k_{+1}} + \frac{1}{k_{-2}K[X]} \quad (3)$$

where $\alpha = K[X]/(1 + K[X])$, and *K*, the equilibrium constant, is given by the ratio of the product of the forward velocity constants to the product of the back velocity constants ($k_{+1}k_{+2}/k_{-1}k_{-2}$). The values of k_{+1} , k_{-2} and k_{+2}/k_{-1} are calculated from the intercepts and slopes of this plot. α was calculated by using $K = 19.97 \text{ M}^{-1}$ (native cytochrome *c*) and $K = 2.58 \times 10^4 \text{ M}^{-1}$ (nitrated cytochrome *c*) as described by do Nascimento (1976).

Results

Effect of imidazole

The reaction of native cytochrome *c* with imidazole at pH 7.0 is monophasic. A plot of the reciprocal of

Fig. 1. Plot of the reciprocal of (α multiplied by the observed rate constant) versus the reciprocal of the imidazole concentration, according to eqn. (3), for the reaction of native cytochrome *c* (a) and for the fast-reacting form of [nitrotyrosyl]cytochrome *c* (b)

(a) The concentration of native cytochrome *c* was 0.2 mM. The buffer used was 0.1 M-sodium phosphate, pH 7.0. The reaction was monitored at 695 nm with a 2 cm light-path at 20°C. (b) The concentration of the protein was 40 μ M. The buffer used was 0.1 M-sodium phosphate, pH 7.0. The reaction was monitored at 612 nm with a 2 cm light-path at 20°C.

Fig. 2. Arrhenius plot for the fast-reacting form of [nitrotyrosyl]cytochrome *c* with imidazole

The protein concentration was 40 μ M. The reaction was monitored at 612 nm with a 2 cm light-path. The buffer used was 0.1 M-sodium phosphate, pH 7.0.

(α multiplied by the observed rate constant) versus the reciprocal of the imidazole concentration in accordance with eq. (3) is shown in Fig. 1(a). The plot is reasonably linear, and in agreement with the results obtained by Sutin & Yandell (1972). The reaction of [nitrotyrosyl]cytochrome *c* with imidazole at the same pH follows biphasic kinetics, and a plot of the reciprocal of (α multiplied by the observed rate constant) versus the reciprocal of the imidazole concentration for the fast-reacting species is shown in Fig. 1(b). In this case, the value of α is practically equal to 1, since K has a large value (do Nascimento, 1976). The slow-reacting component shows no apparent imidazole concentration dependence, and the first-order rate constant was approximately constant for 50–500 mM-imidazole, and was 4.0 s⁻¹. On average, 60% of the absorption change occurs in the fast phase of the reaction.

Table 1. Rate constant for the reaction of native ferricytochrome *c* and [nitrotyrosyl]ferricytochrome *c* with imidazole

The values were calculated from Fig. 1 according to eqn. (3). Details are given in the Materials and Methods section.

	k_{+1} (s ⁻¹)	k_{-2} (s ⁻¹)	k_{+2}/k_{-1} (M ⁻¹)
[Nitrotyrosyl]cytochrome <i>c</i>	87.72 ± 3.07	0.80 ± 0.03	$(1.23 \pm 0.01) \times 10^2$
Native cytochrome <i>c</i>	31.59 ± 0.49	1.31 ± 0.01	0.79 ± 0.002

Fig. 3. Plot of the observed rate versus iminazole concentration for the reaction of [nitrotyrosyl]cytochrome *c*

The concentration of [nitrotyrosyl]cytochrome *c* was $40 \mu M$. The buffer used was 0.1M-sodium acetate, pH4.5. The reaction was monitored at 612nm with a 2cm light-path at $20^\circ C$. The inset shows a van't Hoff plot of $\log$ (initial rate) versus $\log$ (iminazole concentration) for the reaction of iminazole with native cytochrome *c*. Conditions were as described above.

The rate constants obtained from Figs. 1(a) and 1(b) are listed in Table 1. The rate constants are in reasonable agreement with those of Sutin & Yandell (1972), considering that they worked at $25^\circ C$.

The disappearance of the 612nm band of [nitrotyrosyl]cytochrome *c* on addition of imidazole is influenced by temperature. The activation energy calculated from Fig. 2 is $37.78 kJ/mol$.

Effect of iminazole

The reaction of iminazole with [nitrotyrosyl]cytochrome *c* at pH4.5 is second order with respect to substrate. It is monophasic, and a plot of the observed rate constant versus iminazole concentration (Fig. 3) gives a second-order rate constant of $(1.64 \pm 0.04) \times 10^2 s^{-1} \cdot M^{-1}$. On the other hand, the reaction of iminazole with native cytochrome *c* at pH4.5 is first order

Fig. 4. Oscilloscope trace obtained for the reduction of [nitrotyrosyl]cytochrome *c*-imidazole complex with dithionite

The iron(III) of [nitrotyrosyl]cytochrome *c* ($6.0 \mu M$) in 0.5M-imidazole solution was treated with dithionite (5mg/ml) in 0.1M-sodium phosphate buffer, pH7.0. The light-path was 2cm and the wavelength of observation 417nm, with a band-width of 3.4nm. The vertical scale represents a change of 0.02E unit per division and the horizontal scale 5ms per division (a) or 50ms per division (b).

with respect to substrate, as illustrated in the inset to Fig. 3. At 250mM-iminazole, a rate constant of $10 s^{-1}$ was obtained.

Kinetics of decomposition of the complex

The reduction of the imidazole complex of [nitrotyrosyl]cytochrome *c* by dithionite is very fast, and produces an intermediate compound that rapidly decays to a more stable conformation. Fig. 4 represents a typical oscilloscope trace obtained for the experiment. The intermediate species formed in this reaction decays in a first-order fashion. The rates obtained are $5.56 \pm 0.33 s^{-1}$ and $0.49 \pm 0.03 s^{-1}$ for the fast- and slow-reacting component respectively. Again, 60% of the absorption change occurs in the fast phase.

Discussion

In attempts to understand the role of particular residues of cytochrome *c* by kinetic methods a wide variety of specific chemical modifications have been made. Several papers on cytochrome *c* (e.g. Wilson *et al.*, 1973; Brittain & Greenwood, 1975) have indicated that the haem iron atom becomes more susceptible to combination with ligands when the protein is modified.

The comparison of the rate constants for the reaction of cytochrome *c* with imidazole for native and nitrated protein (Table 1) shows that the velocity of the reaction is greatly increased as a result of the

nitration of tyrosine-67. The biphasic character of the reaction of [nitrotyrosyl]cytochrome *c* with imidazole is consistent with the static measurements (do Nascimento, 1976). Form I configuration, the smaller fraction (Schejter *et al.*, 1970), seems to be responsible for the slow concentration-independent step.

Although the values obtained for the limiting rate constant k_{+1} (Table 1) for the reaction of native and [nitrotyrosyl]cytochrome *c* with imidazole are of the same order of magnitude, they are variable by any standard. Creutz & Sutin (1974) pointed out that the rupture of the iron-sulphur bond is not expected to be entirely free from influence of the incoming group. Thus the small variability of the rate constant k_{+1} does not invalidate the mechanism.

At pH 4.5 the disappearance of the high-spin band on reaction with iminazole followed a simple bimolecular mechanism, with a rate constant of $1.64\text{ s}^{-1}\cdot\text{M}^{-1}$. Thus the protein in the high-spin configuration followed a $\text{S}_{\text{N}}2$ reaction mechanism. This is again consistent with the results of Sutin & Yandell (1972), since it suggests that the activated cytochrome *c* can be a protein with an open crevice.

The intermediate reduced species of cytochrome *c* was also obtained by reduction of native cytochrome *c* with Cr^{2+} at alkaline pH (A. J. do Nascimento & C. Greenwood, unpublished work) and consistent with the results obtained by using other reductants, (see, e.g. Land & Swallow, 1971; Pecht & Faraggi, 1972; Lambeth *et al.*, 1973). Our result further strengthens the argument that the secondary changes observed after reduction via a transient intermediate

reduced species are in fact independent of the nature of the reducing agent.

The value for the energy of activation obtained from Fig. 2 shows that there is no significant conformation change associated with the reaction of the [nitrotyrosyl]cytochrome *c* with imidazole.

We thank Dr. C. Greenwood for his helpful comments and suggestions, and Mr. A. C. Thompson for his expert technical assistance. A. J. N. thanks the British Council for a fellowship.

References

- Brittain, T. & Greenwood, C. (1975) *Biochem. J.* **149**, 179–185
- Creutz, C. & Sutin, N. (1974) *J. Biol. Chem.* **249**, 6788–6795
- do Nascimento, A. J. (1976) *Biochem. J.*, **155**, 589–597
- do Nascimento, A. J. & do Nascimento, K. H. (1976) *An. Acad. Bras. Cienc.* **48**, in the press
- Gibson, Q. H. & Milnes, M. (1964) *Biochem. J.* **91**, 161–171
- Lambeth, D. O., Campbell, K. L., Zand, R. & Palmer, G. (1973) *J. Biol. Chem.* **248**, 8130–8136
- Land, E. J. & Swallow, A. J. (1971) *Arch. Biochem. Biophys.* **145**, 365–372
- Margoliash, E. (1954) *Biochem. J.* **56**, 535
- Pecht, I. & Faraggi, M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 902–906
- Schejter, A., Aviram, I. & Sokolovsky, M. (1970) *Biochemistry* **9**, 5118–5122
- Sutin, N. & Yandell, J. K. (1972) *J. Biol. Chem.* **247**, 6932–6936
- Wilson, M. T., Brunori, M., Rotilio, G. C. & Antonini, E. (1973) *J. Biol. Chem.* **248**, 8162–8169