Short Communications

A Comparative Study of some Kinetic and Spectral Properties of Guanidinated and Native Cytochrome *c*

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An investigation of the spectral and some kinetic properties of a chemically modified cytochrome *c* is presented. The kinetics of reduction by chromous ion and ascorbate are shown to be unchanged from that of the native molecule, as are the kinetics of NO binding. The effects of pH on the visible spectrum are discussed in terms of a possible change in the pattern of co-ordination of the molecule with changing pH.

Ever since the characterization of the amino acid composition of cytochrome *c*, a number of workers have sought to explain the function of the protein in terms of the roles played by particular amino acids. The amazing species invariance of certain amino acids (Margoliash & Schejter, 1966) has led a number of workers (Winfield, 1965; Takano *et al.*, 1973; Dickerson, 1974) to propose mechanisms for the main functional role of the protein as a redox carrier which involve some of these residues. One means of investigating the role played by specific amino acids is to modify them by using suitable chemical reagents.

Of the 24 basic amino acids present in cytochrome *c*, 19 are lysine, and it is thought that their positive charge may be important in maintaining conformation (Margoliash, 1961) as well as providing binding sites for the cytochrome oxidase (Smith & Conrad, 1956) and perhaps for some of the other components of the electron-transfer chain.

By treating the molecule with *O*-methylisourea under appropriate conditions it is possible to modify all the lysine residues present (Hettinger & Harbury, 1964). The modification of all the lysine residues would hopefully allow the preparation of a homogeneous product in which the conversion of lysine into homoarginine would produce only minimal structural change. The fact that the chemically modified protein preserves the conformation-sensitive 695nm band seems to support this.

Materials and methods

The guanidination procedure used was essentially that reported by Hettinger & Harbury (1964). However, the *O*-methylisourea hydrochloride used in the preparation was synthesized by the procedure of Schaefer & Zimmerman (1970).

Isoelectric focusing was carried out by applying approx. 20mg of protein to LKB 8101 columns

and focusing for 3 days in the pH range 8-11.0 (Karlsson & Ohman, 1972). Spectrophotometry was performed on a Pye-Unicam (Cambridge, U.K.) SP. 700A instrument, at 20°C. Stopped-flow measurements were carried out in an apparatus essentially that of Gibson & Milnes (1964) as described by Brittain *et al.* (1974).

The enzymic activity of the protein was measured by comparing its ability to restore respiration to cytochrome *c*-depleted mitochondria with that of the native molecule (Aviram & Schejter, 1971). Cytochrome *c*-depleted mitochondria were prepared by the methods of Jacobs & Sanadi (1960) and the rate of respiration was measured with a Rank Brothers (Bottisham, Cambridge, U.K.) oxygen-electrode system.

Gas-equilibrated buffers and CrCl₂ solutions were produced as described by Brittain & Greenwood (1975).

Cytochrome *c* (type III) was used without further purification and was purchased, along with sodium ascorbate, from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Cyanamide, used in the preparation of *O*-methylisourea hydrochloride, was obtained as an aq. 50% (v/v) solution from R. Emanuel Ltd., Wembley, Middx., U.K. NO gas, used in the binding studies, was purchased from Matheson Gas Products, East Rutherford, N.J., U.S.A. All other chemicals were of the highest grade available.

Results and discussion

Table 1 shows a comparison between guanidinated cytochrome *c* and native cytochrome *c* for a number of measured parameters, and the following points emerge. First, the pI changed significantly on modification; this increase, from the already basic value of 10.5 (Tint & Reiss, 1950) for the native mole-

Table 1. *Comparison of some properties of guanidinated and unmodified cytochrome c*

K_a represents the affinity constant obtained by titration and k represents the second-order rate constant for the particular reactions derived from the pseudo-first-order rate plots. E_a is the activation energy of the reduction process measured from variable temperature kinetics. The enzymic activity is given as a percentage relative to the native protein.

	Native cytochrome <i>c</i>	Guanidinated cytochrome <i>c</i>
pI	10.5	11.2
NO binding	$K_a = 9.8 \times 10^3 \text{ M}^{-1}$ $k = 42 \text{ M}^{-1} \cdot \text{s}^{-1}$	$K_a = 1.01 \times 10^4 \text{ M}^{-1}$ $k = 55 \text{ M}^{-1} \cdot \text{s}^{-1}$
Enzymic activity	100%	130%
Ascorbate reduction (pH 6.5)	$k = 12 \text{ M}^{-1} \cdot \text{s}^{-1}$	$k = 8 \text{ M}^{-1} \cdot \text{s}^{-1}$
Reduction at pH 8.0	Biphasic	Biphasic
Cr^{II} reduction (pH 6.5)	$k = 1.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ $E_a = 38.6 \text{ kJ/mol}$	$k = 1.0 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ $E_a = 40 \text{ kJ/mol}$
Effect of pH (6.0 $\rightarrow$ 7.5)	Independent	Independent

Fig. 1. (a) Effect of pH on the α/β region of the visible spectrum of guanidinated cytochrome *c* and (b) titration curve of the 600 nm band of the visible spectrum of guanidinated cytochrome *c* against pH

For (a) spectra were recorded at pH 7.0 (—), 10.5 (---) and 12.3 (— · —) at a protein concentration of $70 \mu\text{M}$ in a 1 cm-path-length cell at room temperature. The buffering systems were Tris-HCl, borate and borate-NaOH respectively, all at 0.2 M. For (b) the protein dissolved in water was used at $17 \mu\text{M}$ in a 4 cm-path-length cell at room temperature (20°C) and was supplemented to $I = 0.3 \text{ mol/l}$ with NaCl. Titration was carried out by adding small portions of 0.2 M-HCl to the alkaline solution of protein.

cule, is in line with the conversion of lysine into the more basic homoarginine. It should be noted, however, that the value of 11.2 obtained for the pI of the modified protein cannot be considered to be completely accurate, as the value obtained lies to one extreme of the pH gradient in the focusing apparatus and hence is liable to error.

The relative enzymic activity presented in Table 1 shows an increase consequent on modification and is in good agreement with the values obtained by Hettinger & Harbury (1964), who determined the succinate oxidase activity manometrically.

The overall results presented in Table 1, coupled with the fact that the guanidinated cytochrome *c* possesses a 605 nm spectral band, suggests only rather minimal functional and structural changes from the native molecule within the physiological pH range.

From the above observations it seems reasonable to conclude that the lysine residues present in cytochrome *c* are not directly involved in any electron transfer from small reducing agents such as Cr^{II} and ascorbate and do not influence ligand binding in the pH range investigated. However, the increased

basic nature of the residues subsequent to modification does appear to enhance enzymic activity, and this has led a number of other workers to suggest that the surface lysine residues of cytochrome *c* may form a recognition site for the other components of the electron-transfer chain (Davies *et al.*, 1963).

The function and spectral properties of the modified protein are maintained over a wide range, but, as Fig. 1 indicates, drastic changes in spectrum are observed at high pH.

Fig. 1(a) shows the spectrum of guanidinated cytochrome *c* at pH 7.0 and 10.5; these spectra are in agreement with those obtained by Hettinger & Harbury (1964), who interpreted this spectral change at high pH as being due to the formation of a hydroxy complex. However, on raising the pH of the solution even higher, to pH 12.3, as shown, a further change occurs in the spectrum, which we would interpret as being due to the co-ordination of the now deprotonated homoarginine-79 to the haem iron.

To investigate further the possible causes of the observed changes in spectrum with pH a titration was carried out to follow changes in the 605 nm band. Fig. 1(b) shows the result of such a titration; two p*K* values are apparent, with values of approx. 9.5 and 11.5. We would interpret these observations as follows. The p*K* at 9.5 is seen as being that normally present in native cytochrome *c*, which represents a transition from type III to type IV (Theorell & Åkerson, 1941), occurring when the ligand methionine-80 is usually replaced by lysine-79. But in guanidinated cytochrome *c* the normally deprotonated lysine residue is now converted into the more basic homoarginine species, which would still possess a positive charge at this pH and so could not co-ordinate in the normal manner. It is thus assumed that an OH⁻ ion from solution takes up position as the sixth co-ordinating species, leading to the spectrum reported in Fig. 1(a). This species remains until a pH of approx. 11.5, when the basic guanidino group deprotonates, so allowing co-

ordination of the 'homoarginine'-79 with consequent change in the spectrum evident as the second p*K*. This interpretation of the observed p*K* value is also supported by some electron-paramagnetic-resonance studies on the modified protein by Morton (1973), who has observed signals at *g* = 2.79, 2.21, 1.76, which he attributes to a monohydroxy complex formed with a p*K* of 9.4.

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