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SPECTROSCOPIC AND REDOX PROPERTIES OF PLANT MITOCHONDRIAL CYTOCHROMES *c*

CARMICHAEL J. A. WALLACE* and DONALD BOULTER†

Département de Biochimie Médicale, Université de Genève, 9, avenue de Champel, 1211 Genève 4, Switzerland; †Department of Botany, University of Durham, Durham DH1 3LE, U.K.

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Key Word Index—*Acer negundo*, *Bixa orellana*, *Pastinaca sativa*, *Chlamydomonas rheinhardtii*; cytochrome *c*; structure-function relationships; spectroscopy; redox potential.

Abstract—Although the sequences of many higher plant cytochromes *c* and the crystal structure of the rice protein have been determined, little is known of their functional properties, compared to the wealth of information available for the vertebrate proteins. We have prepared and examined the cytochrome *c* of three species, and find that the spectral properties and redox potentials are close to those observed for previously characterized vertebrate and fungal proteins. However, the efficacy of electron transfer by plant cytochromes in reconstituted phosphorylation-coupled terminal oxidation chains from rat mitochondria is markedly lower than that exhibited by either vertebrate or yeast cytochromes *c*. We have used both similarities and differences to draw conclusions on structure–function relationships in mitochondrial cytochromes *c*.

INTRODUCTION

Mitochondrial cytochromes *c* have been purified from many species of higher plants, with the principal object of obtaining sequence information for phylogenetic classification [1]. In the course of these studies, some spectral data have been accumulated, but little is known of the other biological or physical properties of these cytochromes.

In contrast, cytochromes *c* from vertebrates and fungi have been the subject of intensive study of the relationship between structure and function in this class of protein [2]. Since the sequences of plant cytochromes demonstrate a number of structural features unique to the Kingdom, it was thought that a closer examination of their properties might yield useful insights into the structure–function relationships general to mitochondrial cytochromes *c*.

Although the principal barrier to such studies is the very low yields of mitochondrial cytochromes obtainable from plant sources [3], we have used techniques developed for the study of semisynthetic cytochromes *c* [4–6], that require only small quantities of protein, to examine the properties of the protein of *Bixa orellana*, *Acer negundo* and *Pastinaca sativa*.

RESULTS

Yields of cytochrome *c* from plant sources are always very low, in the range 10–15 mg from 100 kg of dry seed. This is further reduced if a final ion-exchange step is desirable. Quantities were calculated from the soret band absorbance of solutions of cytochromes. 2.6 mg of the *Acer* protein yielded 1.0 mg after SP-Trisacyl ion-exchange. Since only 0.6 mg of *Bixa* and 0.4 mg of *Pastinaca* cytochromes were available, this step was omitted. The samples used in this work were part of preparations previously made for amino acid sequence studies and had been stored dry at −20° for some years. This method of storage causes no deterioration in vertebrate cytochromes. The state of these samples was checked by examination of the 695 nm absorbance band. This is a very sensitive indicator of the quality of a cytochrome preparation (see below). Only the *Acer* sample did not show a fully developed 695 nm band on removal from storage.

However, the major peak obtained upon SP-Trisacryl cation exchange of the *Acer* protein did have a normal 695 nm band. The ratio of absorbances at 280 and 360 nm was comparable to that of the horse protein, indicating that contamination with non-haem protein was minimal. This was also true of *Pastinaca* cytochrome, but the *Bixa* cytochrome had an elevated 280 nm absorption. Nevertheless, since cytochrome *c* concentrations in redox titrations and biological assays are calculated on the basis of visible wavelength absorptions, these determinations will be insensitive to the presence of non-haem proteins. The spectral data obtained are summarized in Table 1, and show little variation, in terms of peak positions and intensity, from those of the better known vertebrate proteins, thus confirming the observations of Richardson *et al.* [7].

The 695 band, which signals coordination of ferric haem to a methionine sulphur atom, is diagnostic of an intact haem crevice of the protein in state III [2], and disappears with rising temperature, urea concentration or pH in a transition to state IV in all known cytochromes *c*. A mean value for the pK of the transition in

*Author to whom correspondence should be addressed: Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7.

Table 1. Comparison of spectral properties of plant and horse cytochromes *c*

	Horse	*Acer*	*Bixa*	*Pastinaca*
Ferricytochrome α-band	530	530	530	530
Ferrocytochrome α-band	550	550	550	550
β-band	520	520	520	520
Ferricytochrome soret band	410	409	409	409
Ferrocytochrome soret band	416	412	412	414
Ferricytochrome δ-band	360	360	360	358
695 nm band	present	present	present	present
$\frac{A_{550}\text{ ferrocytochrome}}{A_{550}\text{ ferricytochrome}}$	3.4	2.9	3.1	3.4

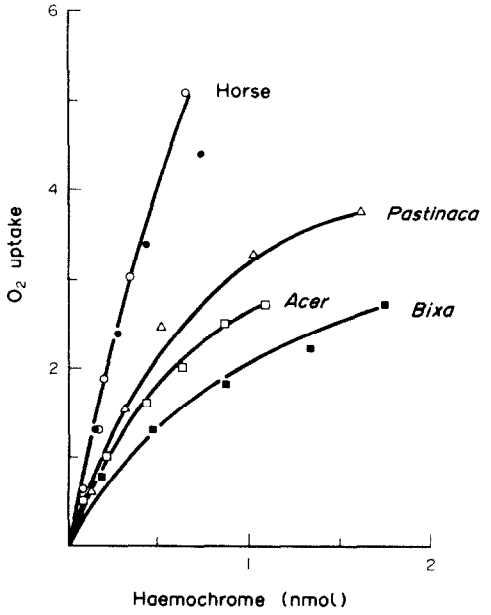

Fig. 1. Biological activity curves for cytochromes *c* with the cytochrome *c* reductase of rat liver mitochondria. Cytochrome *c*-depleted mitochondria were suspended in a buffer containing succinate as electron source, and glucose and hexokinase to remove ATP generated by coupled phosphorylation: sequential additions of cytochrome *c* were made, and O_2 uptake was measured in units of % total dissolved O_2 consumed per minute (data taken from two different mitochondrial preparations).

Acer, of 9.20 ± 0.10, was obtained. Values for *Pastinaca* and *Bixa* obtained from single titrations were 9.15 and 9.20 respectively.

The oxidation-reduction potentials determined for the cytochromes *c* of the three higher plant species were: *Pastinaca* 278 mV, *Acer* 268 mV and *Bixa* 258 mV. These compare with values for horse, of 260 mV and *Saccharomyces*, of 270 mV. The ability to stimulate oxidative phosphorylation, as measured by oxygen consumption, in cytochrome *c*-depleted mitochrondria from rat, is depicted in Fig. 1.

DISCUSSION

The observation that the spectral properties of the plant cytochromes are virtually identical to those of the archetypal horse protein, despite the considerable differences in sequence [8] and tertiary structure [9], reflects the insensitivity of these parameters to changes outside the haem centre itself [2]. Pronounced differences only ensue when the axial ligands or the substituents of the haem *c* are modified.

In contrast, the pK of the disappearance of the 695 nm band with rising pH is very sensitive to changes in protein structure [2,5,10,11]. The pK value signals the stability of the haem crevice to alkaline denaturation, and it is apparent that this parameter is the subject of strong evolutionary conservatism, for the values determined for these three plant species, 9.15–9.20, correspond closely to those of horse, 9.30, tuna, 9.40 or *Saccharomyces*, 9.10. Whether the ligand exchange that produces the spectral transition has physiological significance, or whether the conservatism reflects the need for a precisely controlled deformability of the haem crevice in the electron transfer process, remains an open question [12].

This property of the plant proteins has settled one point of dispute. Lysine 72 cannot provide the replacement during the ligand substitution reaction [10]. Although conserved in all species, the ε-amino group is trimethylated in plants. The group becomes a quaternary ammonium which is permanently ionised and incapable of binding the haem iron.

The values obtained for midpoint oxidation-reduction potentials also reveal evolutionary conservatism. The plant proteins have potentials in the range determined for other mitochondrial cytochromes *c*. The electron transport system of the mitochondrion comprises many carriers, amongst which the redox potentials must harmonise for optimal throughput. For example, the potential of cytochrome *c* is poised between those of its reductase (cytochrome c_1) and oxidase (cytochrome *a*). Shifting that potential by simple structural change away from this optimal value greatly reduces electron transfer rates [13].

Any change in one component must therefore be compensated for by a set of changes elsewhere in the chain, so that the potential of cytochrome *c* has probably been effectively fixed since before the divergence of plants and animals. Another example of this conservatism is the fact that the cytochromes *c* of all vertebrates, and even of *Saccharomyces*, are equally effective when tested in the rat system [6].

However, the biological activities of the plant cytochromes with mammalian reductase are poor compared with that of horse, despite the normal redox potentials they exhibit. Yet they are no more different structurally,

in global terms, from the vertebrate structure than are the yeast proteins. This suggests that there is some specific change in the amino acid sequence that is responsible for this effect. It has been shown that there is a direct relationship between redox potential and the logarithm of biological activity for cytochrome analogues in which the active site is not disrupted [13]. Figure 2 shows that the plant proteins do not conform to this relationship and that the specific change is occurring in that region that constitutes the active site. If we could identify the residue concerned then the nature of the change leading to reduced activity could tell us more about the role of that residue in the mechanism of electron transfer between reductase and cytochrome *c*. Examination of the active site structure of cytochrome *c* (Fig. 3) shows that the binding ring of lysine residues is invariant, but there is one change in the hydrophobic stretch of residues lining the haem edge that could be responsible for the lessened competence.

Residue 83 is Ala or Gly in cytochromes other than those of the higher plants, where it is proline. The role in electron transfer of the region to which it is central is unclear, though it is known to be of considerable importance [6,14,15]. Some of the proposals of electron transfer mechanisms require movement, or at least flexibility, in this region [12,16]. Incorporation of the amino acid proline, with limited rotational flexibility, would restrict the possible conformational changes.

While this substitution might seem the most likely source of the change in biological activity, we cannot positively exclude at this stage the involvement of changes elsewhere in the higher plant structure. However, the cytochrome *c* of the primitive alga *Chlamydomonas rheinhardtii* has a sequence of the higher plant pattern [17] but with alanine at position 83. Preliminary results (Wallace, C. J. A. and Goldschmidt-Clairemont, M.,

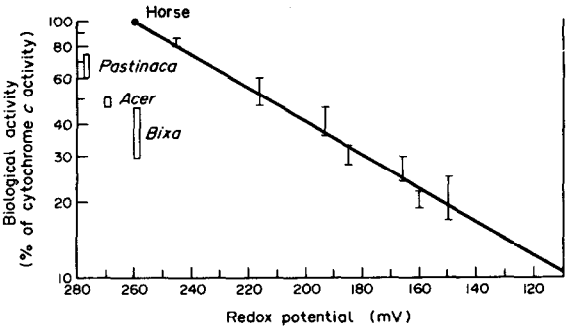

Fig. 2. A comparison of redox potentials and biological activities of cytochrome *c* and analogues. For a series of analogues of the horse protein that do not involve change at the active site (dark bars), there is a direct relationship between the potential and the logarithm of activity with mammalian reductase. These data are taken from ref. [13]. Redox potentials are an average of multiple determinations. The vertical bars represent the range of relative activities observed in several different mitochondrial preparations. The values for higher plant proteins (light bars) do not conform to this relationship.

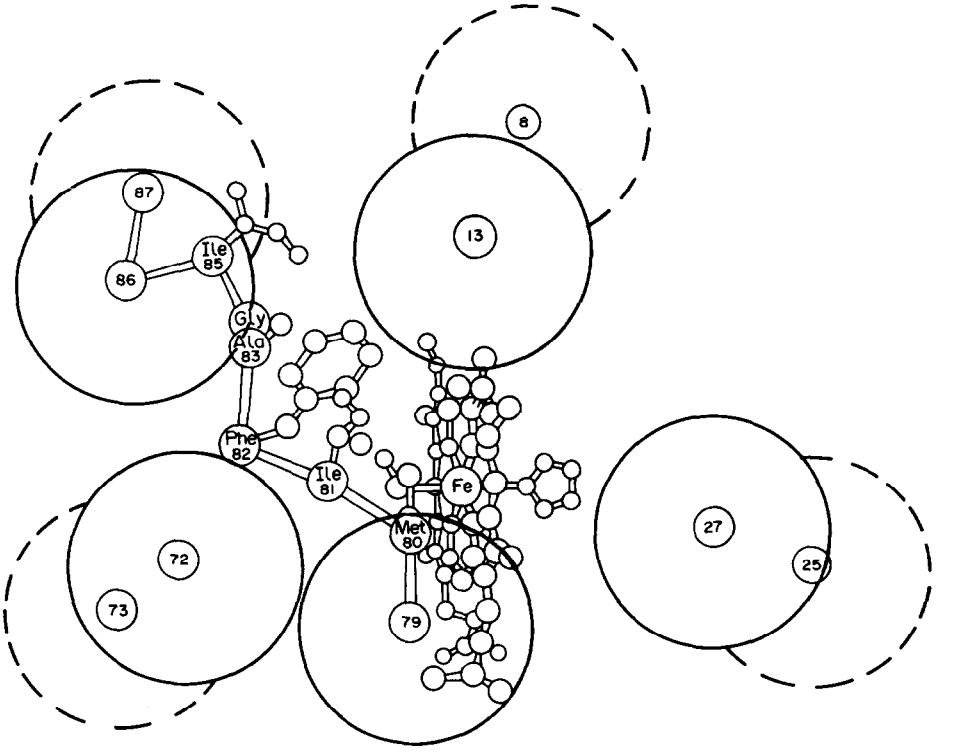

Fig. 3. The active site of cytochrome *c*. The mammalian structure is shown. The lysine residues that bind physiological partners are indicated by their α-carbon atoms and effective radii. They are all absolutely conserved except 8 and 25. Of the amino acids of the haem crevice, 81 (Val in higher plants, Ala in Fungi), 83 (Pro in higher plants, Gly in Fungi) and 85 (Leu in all plants) are variable.

unpublished data) show that while it has lower activity, it does conform to the relationship demonstrated by Wallace and Proudfoot [13]. Semisynthesis [6,14] could be used to insert proline at this position in the horse protein.

EXPERIMENTAL

Preparation and purification of plant cytochromes. Cytochrome *c* was extracted from the germinated seedlings of *Bixa orellana* and *Acer negundo* (box-elder) and from the flowers and seed heads of *Pastinaca sativa* (parsnip) using the techniques of refs [3,7]. Crude preparations were further purified by gel filtration and ion-exchange, as described in ref. [8]. The protein from *Acer* was also chromatographed on SP-Trisacyl cation-exchanger as described in ref. [6].

Visible spectroscopy. Spectra were drawn from 750–325 nm, covering the characteristic absorption bonds of cytochromes *c*, using a Cary 210 spectrophotometer. The weak absorption band at 695 nm was titrated over the pH range 7–11 by addition of 0.1 M NaOH to a cytochrome *c* solution in 0.1 M Pi buffer, pH 7.0 [5].

Oxidation-reduction potentials. Wallace *et al.* [6] have reported a version of the 'method of mixtures', a redox titration using ferro-ferricyanide as the couple, adapted to the small quantities of cytochrome *c* analogues produced by semisynthesis. This method proved suitable for use with the plant proteins.

Biological assay. The mitochondria of rat livers can be depleted of endogenous cytochrome *c*. Electron transfer, which remains coupled to phosphorylation, can be stimulated by the addition of exogenous cytochrome. Sequential additions of plant cytochromes were made to an assay mixture including depleted mitochondria contained in the chamber of a Rank Brothers oxygen electrode, and the resulting stimulation of O_2 uptake measured potentiometrically. The method is described in detail elsewhere [4, 13]: there is evidence that at low concentration of added cytochrome *c*, the reductase to cytochrome *c* step is rate-limiting, so that it is the productivity of the reductase-cytochrome *c* interaction that is being assayed.

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