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BIOLOGICAL SIGNIFICANCE OF METHYLATION OF CYTOCHROMES FROM ASCOMYCETES AND PLANTS

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Abstract—The K_m and V_{max} values characterizing the reaction of baker's yeast *iso*-I-cytochrome *c*, whether trimethylated or not at lysine residue 72, with crude preparations of cytochrome *c* peroxidase, cytochrome *c* oxidase and succinate cytochrome *c* oxidoreductase from *Saccharomyces cerevisiae* are similar. These results, as well as the redox potential values, the auto-oxidability parameters and the circular dichroism spectra, strongly suggest that the biological methylation of yeast cytochrome *c* does not alter its functional properties. The functional characteristics of baker's yeast *iso*-I-cytochrome *c* are similar to those of horse heart cytochrome *c* and yeast *iso*-2-cytochrome *c*.

INTRODUCTION

Cytochromes (cyto) *c* from the ascomycetes *Neurospora crassa*, *Saccharomyces cerevisiae* and *Candida krusei* contain a single residue of ε-*N*-trimethyllysine located at residue 72 in their chemical structure [1, 2]. Cyts *c* from higher plants contain two residues of this amino acid. They were located at residues 72 and 86 in wheat germ cyt *c* and in cyts from *Spinacea oleracea*, *Allium porrum* and *Guizotia abyssinica* [2–5]. Although the position of the ε-*N*-trimethyllysine has not been determined, cyt *c* of *Crithidia fasciculata* (protozoa) contains two residues of this amino acid [6]. On the other hand, methylated amino acids are absent in animal cyt c [7].

Two molecular species of cyts *c*, namely C_I and C_{II} have been isolated from the mi-I (poky) mutant as well as from the wild type of *Neurospora crassa*. These two cyts differ in their chemical structure at residue 72; this position being occupied by trimethyllysine in C_I and by lysine in C_{II} [8, 9]. In *Saccharomyces cerevisiae* also, the methylated and unmethylated forms of *iso*-I-cyt *c* coexist *in vivo* [10].

Recently, Verdière and Lederer have shown that in *Saccharomyces cerevisiae* the methylation of the *iso*-cyts *c* was not linked to the respiratory activity [11]. Their conclusions were based on the observation that the two *iso*-cyts *c* synthesized by two strains of respiratory deficient strains of yeast also possess one residue of ε-*N*-trimethyllysine in their sequences. C_I and C_{II} also were detected not only in the wild type and in the poky mutant of *Neurospora crassa* but also in the respiration-deficient mutants mi-3, cty-I and po-f [8, 9, 12]. Furthermore, as shown by Scott and Mitchell [8], the ratio of C_I to C_{II} in these respiratory deficient strains is similar to that of the wild type at all ages.

However, these results do not exclude the possibility that the trimethylation of lysine residue 72 in these cyts from ascomycetes plays an important role in regulating the activity of these haemoproteins. Such a role, although not yet proved, may be expected from the work of Scott and Mitchell [8]. We decided to investigate such a role for the methylation by the direct comparison of the functional properties of the methylated and unmethylated forms of the *iso*-I-cyt *c* from baker's yeast.

RESULTS AND DISCUSSION

In Table 1, are listed the K_m and the V_{max} values characterizing the reactions of horse heart and baker's yeast cyts *c* with crude preparations of cyt *c* peroxidase, cyt *c* oxidase and succinate cyt *c* oxidoreductase extracted from *Saccharomyces cerevisiae*. The V_{max} values quoted in Table 1 are not absolute ones, since the quantity of pure reductase or oxidase added was not determined. Absolute values are, however, not required for the present work.

Table 1. Michaelis–Menten parameters characterizing the reaction of horse heart and baker's yeast cyts *c* with cyt *c* peroxidase, cyt *c* oxidase and succinate cyt *c* oxidoreductase from *Saccharomyces cerevisiae*

Cytochrome *c*	Peroxidase		Oxidase		Succinate oxidoreductase	
	K_m(μM)	V_{max}*	K_m(μM)	V_{max}*	K_m(μM)	V_{max}*
Horse heart	5.0	19	97.0	68	2.5	20
Iso-I, methylated	3.0	15	21.5	34	0.8	15
Iso-I, unmethylated	3.2	13	22.0	29	0.7	17
Iso-2	3.1	15	23.5	34	0.9	18

* Since crude extracts of cyt *c* peroxidase, cyt *c* oxidase and succinate cyt *c* oxidoreductase were used, the V_{max} values listed in the Table are expressed in arbitrary units.

It may be observed, from Table 1, that all the K_m and V_{max} values obtained for the *iso*-cyts *c* from *Saccharomyces cerevisiae* are nearly indistinguishable. In particular, the methylated and unmethylated forms of yeast *iso*-I-cyt *c* are quite similar. Using horse heart cyt *c* as the substrate, somewhat higher K_m values are observed. This can probably be explained by the heterology of the system used. Nicholls, also, has reported that yeast *iso*-cyts *c* have a lower affinity for mammalian cyt *c* oxidase than the haemoprotein from horse heart [13]. This close similarity could be confirmed by measuring redox potentials. Using the ferrocyanide/ferricyanide couple, redox potentials of 247, 246, 245 and 245 mV were obtained respectively for horse heart, yeast *iso*-I-methylated and unmethylated forms and yeast *iso*-2-cyts *c*. On the other hand, freshly prepared ferrocyts *c* are well known to spontaneously oxidize in solution. For the different cyts we investigated in this study, the auto-oxidation reactions obeyed a pseudo first order kinetic (we performed this measurement at different pH's ranging from 4 to 7.5). For a given pH value, the kinetic constants we measured for horse heart and for the different yeast cyts *c*, again, could not be distinguished.

All these results confirm the close similarity between mammalian and yeast cyts *c*. That the functional properties of *iso*-I-cyt *c* from *S. cerevisiae* is not altered by trimethylation of lysine residue 72 is also strongly suggested. This last conclusion is in good agreement with previous work in which we have shown that methylation of lysine residue 72 of yeast *iso*-I-cyt *c* did not change the micro-environment of the heme as reflected by the circular dichroism spectra in the wavelength range 300–600 nm [14].

The lack of difference between the two forms of yeast *iso*-I-cyt *c* in the reaction with succinate cyt *c* oxidoreductase is not surprising. Indeed, by analogy with the conformation of horse heart cyt *c*, as determined by X-ray diffraction [15], the lysine residue 72 of yeast *iso*-I-cyt *c* may be surmised to be outside the site of interaction with succinate cyt *c* oxidoreductase.

Lysine 72 is an invariant residue in most cyts *c* sequenced so far. Furthermore, a positive charge located at residue 72 has been shown to be important for the interactions between horse heart cyt *c* and cyt *c* oxidase [16]. It is however not evident why trimethylation of *iso*-I-cyt *c* from baker's yeast would be essential for this function. It is true that the trimethyl–ammonium group is a stronger base (higher p*K* value) than the ammonium group. However, both would be cationic at neutral pH.

It seems possible that methylation of cyts *c* from ascomycetes and higher plants has conferred a survival value without modifying greatly their functional properties. This would justify the high energetic cost of such highly specific modification as compared to point mutations.

Peptides containing methylated lysines are enzymatically digested at a slower rate than those containing unmethylated lysines [17]. Also, yeast cyts *c* are much less stable than mammalian ones [18]. From these arguments a role of protection against proteolytic degradation may be surmised for the methylation of cyt *c*.

EXPERIMENTAL

Horse heart cyt *c* (type VI, lot 72C-7160) was purchased from Sigma and used without further purification. Baker's yeast *iso*-1-cyt *c* (the methylated and unmethylated forms of *iso*-1 and *iso*-2) were prepared according to the procedure of ref. [10]. Crude enzyme extracts of cyt *c* peroxidase (cyt *c*: H_2O_2 oxidoreductase—E.C.1.11.1.5.), cyt *c* oxidase *c*: O_2 oxidoreductase —E.C.1.9.3.1.) and succinate cyt *c* oxidoreductase (succinate dehydrogenase—E.C.1.3.99.1.) from baker's yeast were prepared by the procedure described in ref. [19]. The oxidation of cyt *c* by cyt *c* peroxidase was studied at pH 6. (In 50 mM Pi buffer) using an H_2O_2 saturating concn of 125 μM according to ref. [20].

The reaction of cyt *c* with cyt *c* oxidase and succinate cyt *c* oxidoreductase were performed at pH 7 in 0.1 M Pi buffer and followed spectrophotometrically [19]. The assays for the enzymic reduction were carried out in presence of 1 mM KCN and 6 mM succinate. All the enzymatic assays were performed at 20°. Reaction rates are quoted in nmol of reduced or oxidized cyt *c* per min per mg of protein of crude enzymic prep added. Protein concn was determined by the biuret method, after precipitation with 5% TCA [21]. Cristalline BSA served as standard.

The concn of baker's yeast and horse heart cyt *c* were determined spectrophotometrically using the molar extinction coefficients given in ref. [22]. Redox potentials of cyts *c* were determined at 25° at pH 7 (in 0.1 M Pi buffer) using ferrocyanide [22]. The complete oxidation of cyt *c* was achieved by treatment of the protein with K ferricyanide, excess reagent being removed on a column of Sephadex G-25. Ferrocyt was prepared by passing a soln of cyt *c* through a Duolite column. Solns of ferrocyt *c* were prepared shortly before use.

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