EVIDENCE THAT TRIMETHYLATION OF ISO-I-CYTOCHROME *c* FROM *SACCHAROMYCES CEREVISIAE* AFFECTS INTERACTION WITH THE MITOCHONDRION

E. T. POLASTRO, M. M. DECONINCK, M. R. DEVOGEL, E. L. MAILIER, Y. R. LOOZE, A. G. SCHNEK and J. LEONIS

Service de Chimie Générale I, Faculté des Sciences, Université Libre de Bruxelles, 50, Avenue F. D. Roosevelt, B-1050, Bruxelles, Belgique

Received 21 November 1977

1. Introduction

Cytochrome *c* is a protein occurring in all eucaryotic organisms. Its function and gross conformation has remained the same through evolution as indicated by the constancy of properties like its absorption spectrum, biological activity and redox potential [1].

Of the 103 amino acid 'core sequence' common to all types of cytochromes *c* so far sequenced 35 residues are found to be constant [2]. The longest invariant sequence is the undecapeptide comprising residues 70–80. This segment is thought to be very important for the function of the haemoprotein: indeed the sulfur atom of methionine 80 is most probably the sixth ligand to the haeme iron, tyrosine 74 participates to the mechanism of reduction of the haemoprotein by succinate cytochrome *c* oxidoreductase [3] and lysines 72 and 79 appear to be involved in binding with cytochrome *c* oxidase [4].

Yet, the undecapeptide 70–80 is not strictly invariant in all cytochromes *c*, since cytochrome *c* from *Ascomycetes* and higher plants has the lysine 72 ϵ-*N*-trimethylated [5]. The biological role of this posttranslational modification is still unclear and has been proposed [6] that methylation of lysine 72 is more coincidental than specific.

In investigations comparing the methylated and unmethylated forms of iso-I-cytochrome *c* from baker's yeast, we have shown that methylation of lysine 72 does not affect the conformation [7] or the stability towards denaturation by different agents [8]. The two forms showed the same redox potentials, auto-oxidation parameters and capacity to serve as substrates to cytochrome *c* oxidase, cytochrome *c* peroxidase and succinate cytochrome *c* oxidoreductase [9]. Kinetics of digestion by protease A and B of baker's yeast and by trypsin were identical for the two forms (Polastro, E., unpublished results).

We report here that the interaction of cytochrome *c* with the yeast mitochondrion is affected by the methylation of lysine residue 72.

2. Materials and methods

Horse heart cytochrome *c*, type VI, was obtained from Sigma. The fluorescent probe, Dansyl-phosphaditylethanolamine was a kind gift of Dr Tennenbaum. All other chemicals used were of the highest purity available. Methylated and unmethylated forms of iso-I-cytochrome *c* from *Saccharomyces cerevisiae* were prepared as in [10]. Complete oxidation of cytochromes *c* was achieved by treatment of the haemoproteins with potassium ferricyanide. Excess reagent was removed by filtration on a column of Sephadex G-25.

Mitochondria from pig liver or horse heart were

Address correspondence to: E. T. Polastro

obtained by the procedure proposed in [11]. Yeast mitochondria were prepared as in [12].

Endogenous cytochrome *c* was removed as in [13,14].

Protein concentrations were measured by the method in [15].

Cytochrome *c* concentration was determined either by reduced minus oxidized spectra, using the extinction coefficients in [16], or by A_{410} measurement. A Cary 118 spectrophotometer was used.

Cytochrome *c* binding to the mitochondria was assayed at 25°C in 0.225 M mannitol, 50 mM sucrose, 50 mM MOPS buffer, pH 7.2. Binding was measured either according to the 'direct binding' method in [17–19] or by extrinsic fluorescence quenching using Dansylphosphaditylethanolamine as fluorescent probe [19,20].

Fluorescence spectra were obtained on a Hitachi Perkin Elmer MPF 2 A spectrophotometer.

Association constants were determined either by double reciprocal plots or by Scatchard diagrams. In this last representation the following equation [19] was used for the 'direct binding' study:

$$r/A = Kn - Kr$$

were r is no. nmol cytochrome *c* bound/mg mitochondrial protein, A free cytochrome *c* concentration, K association constant ($1/K$ = dissociation constant) and n no. binding sites.

In the fluorescence quenching study, where the amount of cytochrome *c* bound is not directly accessible, we made use of the following assumptions:

(i) The amount of bound cytochrome *c* is always very small with regard to the quantity of cytochrome *c* added, so free cytochrome *c* concentration is assumed to be identical to the analytical quantity of cytochrome *c* added.

(ii) Only 'fluorescent' mitochondria free of cytochrome *c* contribute to the fluorescence intensity.

The assumptions allow one to use the following 'simplified' Scatchard equation:

$$\%\ \text{F.I.Q.}/\ (\text{Cyt } c) = K\ (\%\ \text{F.I.Q.}) - KC$$

where K is association constant, C a constant related to the no. binding sites and %F.I.Q. percentage fluorescence quenching (%F.I.Q. calculated from the equation, $\%\text{F.I.Q.} = 100 \cdot (1 - f/f_0)$, where f_0 and f are, respectively, the fluorescence intensity before and after cytochrome *c* addition; fluorescence intensity was measured using 350 nm for excitation and 500 nm for emission). (Cyt *c*) is the analytical concentration of cytochrome *c* added (μM).

3. Results

Table 1 lists the different values obtained for the

Table 1

Mitochondria \ Cytochrome *c*	Yeast (μM) iso-I methylated	Yeast (μM) iso-I unmethylated	Horse heart (μM)
Yeast	0.04 ± 0.0065 (f) 0.048 ± 0.01 (bd)	0.095 ± 0.005 (f) 0.11 ± 0.014 (bd)	0.122 ± 0.035 (f) 0.1 (bd)
Horse heart	0.085 ± 0.006 (f) 0.077 ± 0.008 (bd)	0.115 ± 0.009 (f) 0.1 (bd)	0.112 ± 0.0015 (f) 0.095 (bd)
Pig liver	0.042 ± 0.009 (f)	0.072 ± 0.013 (f)	Not tested

f, fluorescence quenching method; bd, direct binding method

Dissociation constants ($1/K_a$) of cytochrome *c* interaction with mitochondria cytochrome *c* depleted, in 0.225 M mannitol, 50 mM sucrose, 50 mM MOPS buffer, pH 7.2, at 25°C

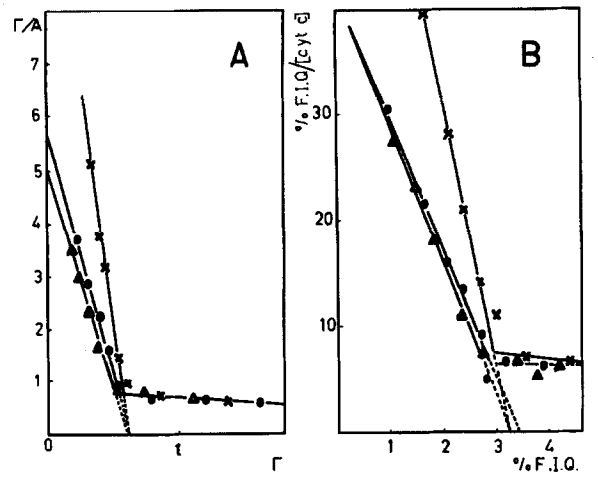

Fig.1. Binding of various cytochromes *c* studied with cytochrome *c*-depleted yeast mitochondria. (X) iso-I- methylated from baker's yeast; (○) iso-I unmethylated from baker's yeast; (△) cytochrome *c* from horse heart. (A) By direct binding method, in 0.225 M mannitol, 50 mM sucrose 50 mM MOPS buffer, pH 7.2, at 25°C. *r*, nmol bound cytochrome *c*/mg mitochondrial protein; *A*, concentration of free cytochrome *c* (μM). (B) By the fluorimetric method, the mitochondria were incubated in 10 μM Dansyl-phosphaditylethanolamine in the same buffer as described above. Fluorescence intensity was measured using 350 nm for excitation and 500 nm for emission. %F.I.Q. is percentage fluorescence quenching (obtained by the formula %F.I.Q. = $100 \cdot (1-f/f_0)$ where f_0 and f are, respectively, the fluorescence intensity before and after cytochrome *c* addition); [cyt *c*] analytical concentration of cytochrome *c* added (μM).

dissociation constant of cytochromes *c* with the mitochondria isolated from yeast, pig liver and horse heart. The data are all the result of an average of 3–5 independent experiments.

Figure 1 shows a Scatchard plot of cytochrome *c* interaction with yeast mitochondrion, either by the 'direct binding' method (A) or by fluorescence quenching measure (B).

The fluorimetric study is somewhat gross, given the assumptions introduced to handle the experimental data, but also because the reabsorption of emitted light by the increasing amounts of cytochrome *c* added is not taken into account. We did not find correction by the formula of [21] suitable in this case, because of the turbidity of the solution due to the suspension of mitochondria.

However, since our aim was only to compare the binding constants and not to determine analytical or absolute values, and since these systematic errors were constant for all kinds of cytochromes investigated here, the relative values obtained by the fluorimetric method can be considered satisfactory for comparison studies.

Whatever the method used, the methylated form of baker's yeast appears to interact with the yeast mitochondrion 2–3-times more strongly than the unmethylated form or than the horse haemoprotein.

This difference is significant, given the statistical dispersion, between 20% and 30%.

The difference of dissociation constants between methylated and unmethylated yeast iso-I-cytochrome *c* is much less obvious when interacting with heterologous mitochondria. Such an observation could perhaps suggest that methylation of cytochrome *c* has become useless in animals and therefore has been deleted during evolution.

We have of course checked that the differences in dissociation constant were not merely an 'artifact' due to different oxidation states of the cytochrome *c* [18,19].

The number of binding sites of high affinity can be estimated directly by the direct binding method, or indirectly by the fluorescence quenching. It seems to be identical for the three cytochromes *c* studied, some little variations existing, however, according to the origin of mitochondria.

No significative differences, either in the number of binding sites, or in the dissociation constant, can be noticed in the low affinity binding zone [17].

4. Discussion

The data reported here strongly suggest that the methylation of lysine 72 of iso-I-cytochrome *c* from *Saccharomyces cerevisiae* increase the affinity of the protein towards the mitochondrion.

This conclusion is supported by two observations:

1. The work in [18] has suggested that cytochrome *c* binds to the mitochondrial membrane at the site where methionine 65 is located. This residue can be surmised, from X-ray diffraction studies, to be spatially localized not very far from residue

72 [22]. Moreover lysine 72 is thought to participate in interactions with cytochrome *c* oxidase [4].

2. The studies [23] on the poky mutant (mi-I) of *Neurospora crassa*, containing up to 16-times the normal content of cytochrome *c* and having a very high proportion of unmethylated cytochrome *c*, have shown that the ratio of the methylated and unmethylated forms of cytochrome *c* was 0.55 in the whole cell but that only the methylated form was extractable from the mitochondrial fraction.

These authors have suggested that methylation of cytochrome *c* could reflect the binding of the haemoprotein to the mitochondrial matrix, or that the methylated form is bound to this organite more tightly, and does not leak out during purification of mitochondria [23].

Our results are in good agreement with this last hypothesis, which could be biologically significant since cytochrome *c* is synthesized outside the mitochondrion, in the cytoplasm. By the mass action law, methylation of lysine 72, also occurring in the cytoplasm [24], increasing the affinity of the haemoprotein for the mitochondrion, could reduce the amount of cytochrome *c* necessary for cellular respiration.

The role of the methylation of cytochrome *c* could thus be to facilitate its binding to the mitochondrion. From a molecular point of view, the trimethylation of lysine not only conserves and makes permanent a positive charge on the ε-N-amino function of this amino acid, but also creates an additionnal external hydrophobic area [1]. This center could be useful in either hydrophobic or ionic interactions with the mitochondrial membrane.

Acknowledgements

Authors are grateful to Professor A. Sels for many discussions, to Professor R. Kram for critical reading of the manuscripts and to Professors A. O. Barel and E. Schramm for permission to use their Cary 118 spectrophotometer. Thanks are due to Professor A. Burny and to Drs G. Huez and G. Marbaix for the loan of their Eppendorf centrifuge. E. Polastro is Aspirant du Fonds National de la Recherche Scientifique. M. Devogel is an IRSIA fellow.

References

[1] Smith, E. L. (1970) in: The Enzymes (Boyer, P. D. ed) Vol. 1, pp. 267–337, Academic Press, New York.
[2] Dayhoff, M. O., Park, C. M. and McLaughlin, P. J. (1972) in: Atlas Protein Sequence Struct. 5, pp. 7–16, Washington Nat. Biomed. Res. Found.
[3] Margoliash, E., Ferguson-Miller, S., Tulloss, J., Kang, C. H., Feinberg, B. A., Brautigan, D. L. and Morrison, M. (1973) Proc. Natl. Acad. Sci. USA 70, 3245–3249.
[4] Staudenmayer, N., Ng, S., Smith, M. B. and Millet, F. (1977) Biochemistry 16, 600–604.
[5] Cantoni, G. L. (1975) Ann. Rev. Biochem. 44, 435–451.
[6] Pettigrew, G. W. and Smith, G. M. (1977) Nature 265, 661–662.
[7] Looze, Y., Polastro, E., Gielens, C. and Léonis, J. (1976) Biochem. J. 157, 773–775.
[8] Polastro, E., Looze, Y. and Léonis, J. (1976) Biochim. Biophys. Acta 446, 310–320.
[9] Polastro, E., Looze, Y. and Léonis, J. (1977) Phytochemistry 16, 639–641.
[10] Foucher, M., Verdière, J., Lederer, F. and Slonimski, P. P. (1972) Eur. J. Biochem. 31, 139–143.
[11] Chance, B. and Hagihara, B. (1963) Proc. 5th Congr. Biochem., Moscow 1961 (Sissakian, A. N. M. ed) Vol. 5, pp. 3–37.
[12] Lang, B., Burger, G., Doxiadis, I., Thomas, D. Y., Bandlow, W. and Kandelvitz, F. (1977) Anal. Biochem. 77, 110–121.
[13] Jacobs, E. E. and Sanadi, D. R. (1960) J. Biol. Chem. 235, 531–534.
[14] Boveris, A., Erecinska, M. and Wagner, M. (1972) Biochim. Biophys. Acta 256, 223–242.
[15] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. (1951) J. Biol. Chem. 193, 265–275.
[16] Margalit, R. and Schejter, A. (1970) FEBS Lett. 6, 278–280.
[17] Williams, J. N. and Thorp, S. L. (1970) Arch. Biochem. Biophys. 141, 622–631.
[18] Vanderkooi, J., Erecinska, M. and Chance, B. (1973) Arch. Biochem. Biophys. 157, 531–540.
[19] Vanderkooi, J., Erecinska, M. and Chance, B. (1973) Arch. Biochem. Biophys. 154, 219–229.
[20] Shechter, E., Gulik-Krzywicki, T., Azerad, R. and Gros, C. (1971) Biochim. Biophys. Acta 241, 431–442.
[21] Burstein, E. A. (1968) Biofizica 13, 433–457.
[22] Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A. and Margoliash, E. (1971) J. Biol. Chem. 246, 1511–1535.
[23] Scott, W. A. and Mitchell, H. K. (1969) Biochemistry 8, 4282–4289.
[24] Nochumson, S., Durban, E., Kim, S. and Paik, W. K. (1977) Biochem. J. 165, 11–18.