COMPLEX FORMATION BETWEEN YEAST CYTOCHROME b_2 AND RAT LIVER MITOCHONDRIAL INNER MEMBRANE

G. CREMEL and A. WAKSMAN+

Centre de Neurochimie du CNRS, Institut de Chimie Biologique, 11 Rue Humann, 67085 Strasbourg Cedex

and

P. PAJOT++

Centre de Génétique Moléculaire du CNRS, 91000, Gif-Sur-Yvette, France

Received 7 January 1977

1. Introduction

In previous experiments, Waksman and Rendon [1] demonstrated that the localization of some proteins and enzymes in the different submitochondrial compartments seemed to be a function of the environment of the organelle. This phenomenon which can best be described as intramitochondrial large amplitude protein movement appeared to be reversible, specific and lipid-dependent [2].

One intriguing question raised by this phenomenon was the possible functional role played by the mobile proteins whose localization depended on the environment. If one considers the most characteristic property of proteins, which is to recognize or to be recognized by ligands, it is possible to conceive of two extreme situations: either the ligand is or is not an enzyme substrate.

In bacteria flavin-linked D-lactate dehydrogenase has been shown to reconstitute both respiration and transport of D-lactate into vesicles obtained from bacterial mutants naturally deprived of lactate dehydrogenase activity [3].

Yeast cytochrome b_2 constitutes a good model system in our case. Cytochrome b_2 (EC 1.1.2.3) [4] is a mitochondrial enzyme known to occur only in yeasts. This enzyme catalyses the transfer of electrons from L-lactate to cytochrome *c* and, in vivo, allows the cells to respire and to grow, even in the presence of antimycin A when L-lactate is the source of energy [5]. Experimentally, its presence in the inner mitochondrial membrane can be easily detected. Thus, if binding of this yeast enzyme to rat liver mitochondrial membrane occurs, one might hope to be able to detect this association and this would suggest that a well defined mitochondrial function might depend on the transitory situation of protein.

2. Materials and methods

Mitochondria were isolated as mentioned by Waksman and Rendon [1] except that the organelles were only washed twice. Mitoplasts were prepared according to the digitonin method [7]. By spectrophotometry at low temperature, it can be checked that the treatment has no effect on the cytochrome *c* content of the inner membrane preparation (fig.1). Initial mitochondrial concentration was always 25 mg protein/ml. Results were referred to that initial concentration.

Oxygen consumption was measured in a medium containing mitoplasts at a concentration of 2.5 mg

+ Maître de Recherche au CNRS, to whom requests for reprints should be addressed

++ Chargé de Recherche au CNRS

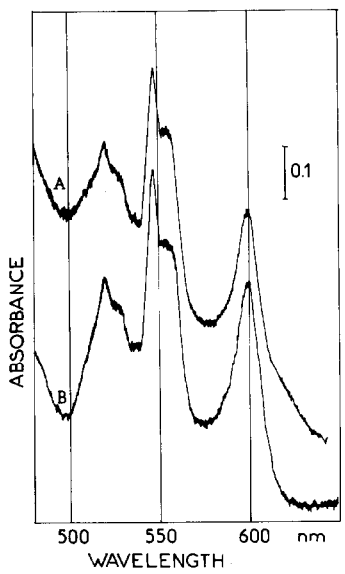

Fig.1. Low temperature spectra of rat liver mitochondria (A) and mitoplasts prepared by the digitonin treatment (B). The particles were suspended in 0.25 M sucrose and the spectra recorded under the conditions described by Claisse and Pajot [10].

of initial protein, various concentrations of cytochrome b_2 in 0.25 M sucrose, 5 mM $MgCl_2$, 20 mM KCl and 10 mM phosphate buffer, at pH 7.4, in a final volume of 1.7 ml, in a Gilson type oxygraph equiped with a Clark electrode. Two types of flavocytochrome b_2 were used. The enzyme prepared from commercial baker's yeast (*Saccharomyces cerevisiae*) was isolated according to the method of Appleby and Morton [5] with the modification of Spyridakis et al. [8]. The enzyme isolated from *Hansenula anomala* was extracted according to Baudras and Spyridakis [9] with the modification of Naslin and Labeyrie (unpublished). Cytochrome b_2 fixation to mitoplasts was followed on 1 ml of mitoplast preparation obtained from rat liver mitochondria (initial protein concentration, 25 mg/ml incubated at 30°C for 5 min in the presence of increasing concentrations of cytochrome b_2 (from 0–35 nmol) in a 10 mM phosphate buffer at pH 7.2. Thereafter the mixtures were centrifuged at 57 000 × g for 15 min at 4°C in R 40 rotor in a model L 50 Beckman type centrifuge. Pellets and supernatans were recovered and assayed for enzymatic activity.

Cytochrome b_2 contents were determined enzymatically by following the rate of reduction of ferricyanide at 418 nm in 10 mM phosphate buffer, at pH 7.2, in the presence of 10^{-3} M ferricyanide and 20 mM L-lactate.

3. Results and discussion

3.1. *Oxidation of L-lactate by rat liver mitoplasts in presence of cytochrome* b_2

A typical respiration curve obtained with mitoplasts, incubated in the presence of L-lactate and cytochrome b_2 is shown in fig.2 (final spec. act. 14 nmol/min/mg). A control in the absence of cytochrome b_2 did not show any noticeable oxidation.

The observed respiration is not inhibited by antimycin A at usual inhibitory concentrations (0.2 μg/mg protein) but is inhibited by 1 mM potassium cyanide. This agrees with the inhibition of respiration on L-lactate of *S. cervisiae* mitochondria [6].

These data suggest the possibility that a complex is formed between yeast cytochrome b_2 and rat liver mitoplasts in which this enzyme would be located functionally somewhere along the respiratory chain

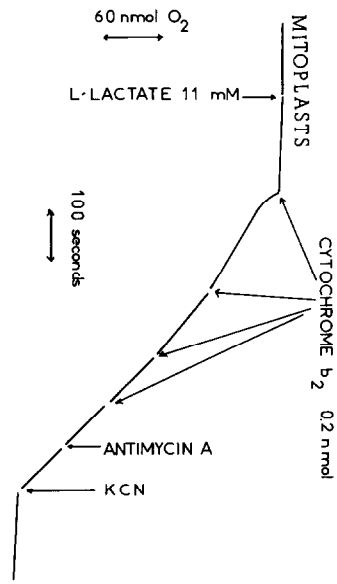

Fig.2. Respiration pattern of L-lactate by rat liver mitoplasts in the presence of yeast cytochrome b_2 measured by oxygraphy. Concentration of mitoplasts in oxygraph chamber, 2.5 mg of initial protein.

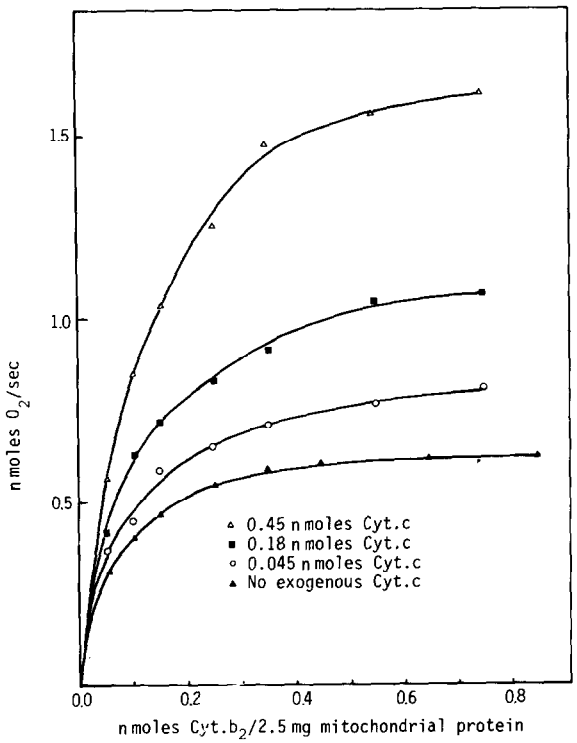

Fig.3. Respiration of L-lactate by rat liver mitoplasts as a function of increasing concentrations of cytochrome *c* and yeast cytochrome b_2.

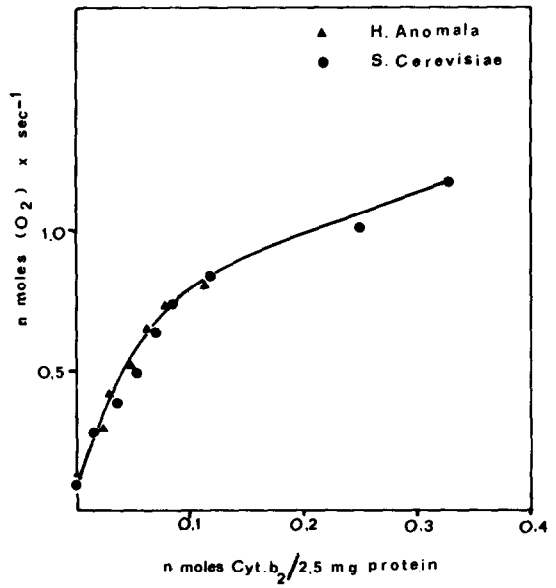

Fig.4. Respiration of L-lactate by rat liver mitoplasts as a function of increasing concentrations of *Saccharomyces* or *Hansenula* cytochrome b_2.

after the antimycin A binding site, precisely as with cytochrome b_2 in *S. cerevisiae.*

Dependency of the respiration on L-lactate as a function of both cytochrome *c* and yeast cytochrome b_2 in rat liver mitoplasts is presented in fig.3.

This figure shows (a) that, at the various concentrations of cytochrome *c* and b_2 tested, the oxidation kinetics reveal a saturation phenomenon. (b) That regardless of the concentration of the exogenous cytochrome *c* added the curves level off at a concentration of about 0.1–0.2 nmol cyrochrome b_2/ 2.5 mg mitochondrial protein.

This suggests the possible existence of an interaction area for the cytochrome b_2 in rat liver mitoplasts. This is further confirmed with cytochrome b_2 from *Hansenula nomala* [9] (a 6-fold more active enzyme). Here again respiration of rat liver mitoplasts in the presence of L-lactate is 'induced'. The respiration curve is identical to the one obtained with cytochrome b_2 from *S. cerevisiae* (fig.4), strongly suggesting the formation of a defined functionally active complex between cytochrome b_2 from either source and rat liver mitoplasts.

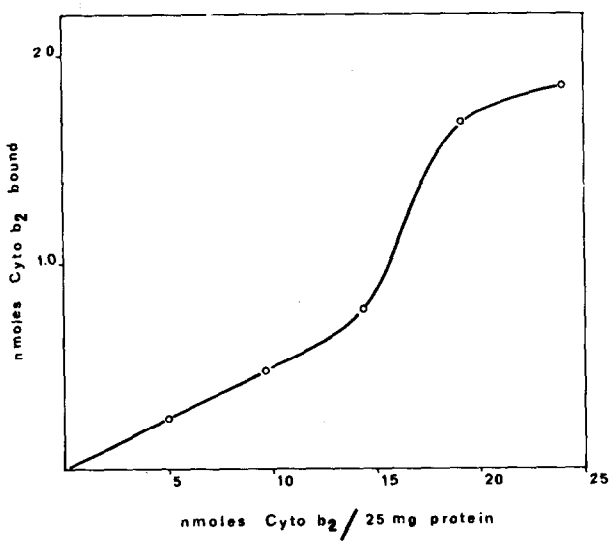

Fig.5. Binding of cytochrome b_2 to rat liver mitoplasts.

3.2. *Binding of cytochrome b_2 to rat liver mitoplasts*

More direct evidence for complex formation between cytochrome b_2 and rat liver mitoplast is provided by following the binding of cytochrome b_2 to mitoplast (fig.5). This binding appears as a sigmoïdal type phenomenon suggesting a possible cooperativity between this enzyme and the mitoplastic membrane and brings about a direct argument in favor of the existence of fixation sites. The absence of binding of the yeast enzyme at low concentration was checked by using, instead of the *Saccharomyces cerevisiae* enzyme, cytochrome b_2 isolated from *Hansenula anomala* which is endowed with a 6-fold higher activity and thus allows one to detect lower enzyme contents. When less than 2.5 nmol of cytochrome b_2 is added to 25 mg of whole mitochondrial protein, no binding could be detected, all the activity being recovered in the supernatant. This indicates that binding rather than enzymatic activity is the limitating step in L-lactate respiration with rat liver mitoplasts.

However, the interaction of cytochrome b_2 with the rat liver mitochondrial membranes is superficial, since an antiserum against the yeast enzyme inhibits the complex.

Further localisation of yeast cytochrome b_2 in or on rat liver mitoplasts might bring some information on the nature of the interactions involved in this complex.

Acknowledgements

We wish to acknowledge the skilful technical assistance of Mrs R. L. Rendon, E. Bogner and N. Cremel. Part of this work has been supported by the 'Institut National de la Santé et de la Recherche Médicale', grant No. 73.4.081.6 and by the 'Fondation pour la Recherche Médicale Française'.

References

[1] Waksman, A. and Rendon, A. (1974) Biochimie 56, 907–924.
[2] Cremel, G., Hubert, P. and Waksman, A. (1975) Physiol. Chem. Phys. 7, 487–494.
[3] Reeves, P., Hong, J-S. and Kaback, H. R. (1973) Proc. Natl. Acad. Sci. USA 70, 1917–1921.
[4] Appleby, C. A. and Morton, R. K. (1954) Nature 173, 749–752.
[5] Appleby, C. A. and Morton, R. D. (1959) Biochem. J. 71, 492–499.
[6] Pajot, P. and Claisse, M. L. (1974) Eur. J. Biochem. 49, 275–285.
[7] Schnaitman, S. C., Erwin, V. G. and Greenawalt, J. W. (1967) J. Cell. Biol. 32, 719.
[8] Spyridakis, A., Nashin, L. and Labeyne, F. B. (1971) Biochimie 53, 195–205.
[9] Baudras, A. and Spyridakis, A. (1971) Biochimie 53, 943–955.
[10] Claisse, M. L. and Pajot, P. (1974) Eur. J. Biochem. 49, 49–59.