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# Phosphate Transport in Yeast Mitochondria: Purification and Characterization of a Mitoribosomal Synthesis Dependent Proteolipid Showing a High Affinity for Phosphate†

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ABSTRACT: It is possible to obtain from yeast mitochondria a proteolipid able to bind phosphate, by two different procedures. One of them, generally used for lipid extraction, leads to the preparation of a more active crude proteolipid. This crude proteolipid has been purified by various chromatographic procedures and the active fraction, in phosphate binding, is always associated with cardiolipin. Its molecular weight seems to be close to 10 000. The phosphate binding shows ligand saturation behavior and is inhibited by arsenate and *N*-ethylmaleimide; succinate is noninhibitory. This protein seems to be dependent on the mitoribosomal synthesis since it is not present in mitochondria of mutant "petite colonie" and its amount largely decreases in mitochondria from yeast grown in the presence of chloramphenicol. It is possible to extract a proteolipid from the oligomycin sensitive ATPase, showing the same activity and properties. The hypothesis that this proteolipid acts as a part of the $P_i$ carrier and constitutes the oligomycin-sensitive ATPase complex is discussed.

Recently the effects of some physiological or genetic manipulations on yeast mitochondria were studied with regard to phosphate transport (Arselin de Chateaubodeau et al., 1976; Rigoulet et al., 1977). It was shown that the phosphate carrier assembly was under the dual dependence of the cytosolic and the mitochondrial protein synthesis. For instance, saturation kinetics for the phosphate transport was detected only in mitochondria isolated from cells in which the mitochondrial protein synthesis was functional.

Products of the mitoribosomal protein synthesis are known to have a high degree of hydrophobicity and to be extracted only with detergent or organic solvents (Tzagoloff et al., 1973; Kadenbach & Hadvary, 1973a; Kuela et al., 1975; Brambl and Handshin, 1976). Several studies have been reported in which these mitochondrial proteins, defined as proteolipid (Folch-Pi & Lees, 1971), were extracted with mixtures of chloroform–methanol. Some of these products are well defined and characterized: the DCCD binding protein (Cattell et al., 1971; Stekhoven et al., 1972); a subunit of the oligomycin-sensitive ATPase (Tzagoloff et al., 1973); a low molecular weight proteolipid in rat liver mitochondria (Dianoux et al., 1976). Particularly, Kadenbach & Hadvary (1973a) have shown that a chloroform extract of rat liver mitochondria was able to bind specifically phosphate.

Studies described in this paper were initiated to gain better understanding of this proteolipid in yeast mitochondria. Purification procedures, some properties, and the origin of the proteolipid able to bind phosphate are investigated. The possibility that this proteolipid could be a part of the phosphate carrier system and/or of the OSATPase is discussed. Preliminary data of these studies have already been reported (Napias & Guérin, 1976; Guérin et al., 1977).

## Experimental Procedures

### *Materials*

*Saccharomyces cerevisiae,* yeast foam diploid wild strain, is grown aerobically on a 2% galactose medium, supplemented

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or not with chloramphenicol (4 g/L); the isolation procedure of mitochondria has been described previously (Arselin de Chateaubodeau et al., 1976). Gal[1] mitochondria refer to yeast grown without chloramphenicol, and CAP mitochondria to yeast grown in the presence of chloramphenicol. A mutant $\rho^-$"petite colonie" from this strain is also used ("petite" mitochondria).

*Experimental Methods*

*ATPase Preparation.* The oligomycin-sensitive ATPase is isolated from mitochondria and purified according to Ryrie (1975). The ATPase activity is measured according to Somlo (1968) at 30 °C and pH 8.5.

*Proteolipid Extraction.* Two methods were used: the first one follows Kadenbach & Hadvary (1973a); the second one uses the lipid extraction procedure of Bahl et al. (1970).

Method I. Mitochondria (400 mg) are precipitated with 10% trichloroacetic acid and heated for 1 h at 70 °C; after washing, the precipitate is dissolved in 40 mL of a 5% $NaDodSO_4$, 5% $\beta$-mercaptoethanol, 10 mM $\gamma$-aminobutyric acid, 50 mM Tris-HCl (pH 8.5) buffer. This solution is extracted with 160 mL of chloroform–methanol (2:1, v/v). The organic phase is washed with 0.73% NaCl and evaporated to dryness. The solid residue is dissolved in chloroform.

Method II. Mitochondria (400 mg) are heated at 50 °C twice directly in 100 mL of chloroform–methanol (1:2, v/v). After filtration, the combined extracts are evaporated to dryness and the residue is dissolved sequentially with 100 mL of chloroform, 94 mL of water, and 6 mL of methanol. After mixing and separation of the phases, the aqueous phase is reextracted with 40 mL of chloroform; the combined chloroformic phases are washed with water and evaporated to dryness, and the residue is dissolved in chloroform.

In both cases the chloroform extracts are referred to as crude proteolipid.

*Proteolipid Purification.* The crude proteolipids are purified by various chromatographic procedures: (1) by chromatography on a column of Sephadex LH-20 equilibrated and eluted with chloroform–methanol–acetic acid–water (20:20:1:1) according to Kadenbach & Hadvary (1973b); (2) by chromatography on a column of DEAE-cellulose acetate according to Allen et al. (1966); sequential elution of phospholipids is obtained by mixtures of chloroform–methanol (49:1; 19:1; 9:1; 0:1), v/v, and then with chloroform–methanol (2:1) containing increasing concentrations of ammonium acetate (3.3 g/L; 10 g/L); (3) according to Steiner & Lester (1972) by two-dimensional chromatography on SG 81 paper impregnated with 2% EDTA (pH 7.2) with the first dimension being chloroform–methanol–water–ammonia (66:16:0.9:3) and the second dimension being chloroform–methanol–water–acetic acid (32:4:1:5).

*Phosphate Binding Test.* The phosphate binding activity is measured according to Kadenbach & Hadvary (1973b) on the crude proteolipid and on the purified fractions after a complete elimination of the acetic acid present in the solvent. The proteolipid (3 to 20 $\mu$g of protein) in solution in 0.8 mL of chloroform (analytical grade) is vigorously mixed for 1 min on a cyclomixer (50 cycles $s^{-1}$) with 0.4 mL of a solution of [$^{32}$P]phosphate (various molarities), 50 mM Tris-HCl (pH 7.3) in chloroform-saturated water–methanol (1:1). After centrifugation, 0.5 mL of the chloroformic phase is taken out and its radioactivity determined in 10 mL of a solution of 4 g of Omnifluor/L of toluene. In all the cases, results are corrected for quenching and radioactivity decrease.

In order to test the thiol reagent sensitivity, 50 $\mu$L of 100 mM NEM, dissolved in the same buffer, is mixed vigorously for 3 min before addition of the [$^{32}$P]phosphate solution.

*Other Methods*

*Organic phosphorus* is assayed by a procedure allowing the determination of 0.5 $\mu$g of phosphorus (Napias, 1975): chloroformic solutions of lipids are evaporated to dryness; 0.1 mL of sulfuric acid and 0.25 mL of nitric acid are added sequentially; the test tubes are heated at 250 °C until the disappearance of nitrous vapors and total bleaching. After cooling, 1.1 mL of distilled water is added and tubes are heated for 15 min at 100 °C. After cooling, 0.6 mL of 5% ammonium molybdate, 0.3 mL of 20% sodium sulfite, and 0.3 mL of 0.5% hydroquinone (pH 3) are added sequentially. The intensity of the blue color appearing after 10 min of heating at 40 °C is determined at 720 nm after 30 min.

*Phospholipid analysis* is done by thin-layer chromatography on silica gel G in chloroform–methanol–water (80:25:4) and revealed with Kostetsky's reagent (Vaskovsky & Kostetsky, 1968).

*Protein determination* is done according to Lowry et al. (1951) after filtration of the reaction mixtures on Millipore filters.

*Gel electrophoresis* is performed according to Cattell et al. (1971) for the proteolipid and according to Weber and Osborn (1969) for the ATPase.

## Results

*I. Extraction and Purification.* Crude proteolipids were extracted from yeast mitochondria by both methods described in the Experimental Procedures section. Both procedures led to the isolation of a chloroform extract able to bind [$^{32}$P]-phosphate. But differences appeared in the properties of these chloroform extracts and in the purified proteolipids (Table I): the ratio phospholipids/protein in the chloroform extract was about 10 times higher when method II was used. At the same time, the phosphate binding activity of the crude proteolipid obtained by the second method was five times higher than when using Kadenbach's method. This higher activity was also found after purification of the proteolipid on Sephadex LH-20, pointing out a possible role of the relationship between phospholipids and protein.

Consequently method II was chosen to study the purification and the characterization on the proteolipid.

First the chloroform extract was chromatographed on a column of Sephadex LH-20, according to Kadenbach & Hadvary; but in contrast to their method, the chloroform extract was not preloaded with [$^{32}$P]phosphate. Then, the binding activity of all the isolated fractions could be measured.

Since proteins and phospholipids absorb ultraviolet, detection by monitoring with UV light (280 nm) was ineffective. Therefore, separate protein and lipid phosphorus analyses were done on the effluent. Figure 1A shows a typical elution profile: several peaks of proteins appeared as determined by Lowry's method. Protein fractions were pooled according to Figure 1A (pooled fractions 1 to 7). A qualitative analysis of the phospholipid content of each fraction, performed by thin-layer chromatography, shows a sequential elution of the various kinds of phospholipids: cardiolipin, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and phosphati-

[1] Abbreviations used: NEM, *N*-ethylmaleimide; CAP, chloramphenicol; Gal, galactose; OS-ATPase, oligomycin sensitive ATPase; $P_i$, inorganic phosphate; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; CL, cardiolipin; $NaDodSO_4$, sodium dodecyl sulfate; DCCD, dicyclohexylcarbodiimide.

TABLE I: Comparative Results Obtained by Both Extraction Procedures.[a]

| Origin of the proteolipid | Method I | | Method II | | |
|---|---|---|---|---|---|
| | Gal mitochondria[b] | CAP mitochondria[b] | Gal mitochondria | CAP mitochondria | Mitochondria from "petite"[b] |
| No. of experiments | 3 | 2 | 4 | 2 | 2 |
| Crude proteolipid | | | | | |
| mg of phospholipid/mg of protein | 3.5 | — | 33 | 57 | 19 |
| $N^c$ | 0.4 | 0.15 | 2 | 0.08 | 0.05 |
| Purified proteolipid (after Sephadex LH-20) | | | | | |
| $N^c$ | 2-5 | 2.2 | 8-15 | 6 | 0.08 |
| Inhibition by NEM in % | 75 | 75 | 78 | 68 | |

[a] Phosphate binding test was performed with 20 to 50 μg of protein in crude proteolipid and 5 to 7 μg of protein in the purified proteolipid. [Phosphate] = 10 μM. Specific activity = $8 \times 10^5$ dpm/nmol of P. Mixing time = 1 min. [b] Gal mitochondria: isolated from yeast grown on 2% galactose. CAP mitochondria: isolated from yeast grown on 2% galactose plus 4 g/L chloramphenicol. "Petite"-mitochondria: isolated from a mutant "petite colonie" grown on 2% galactose. [c] $N$, nmol of bound $P_i$/mg of protein of the proteolipid.

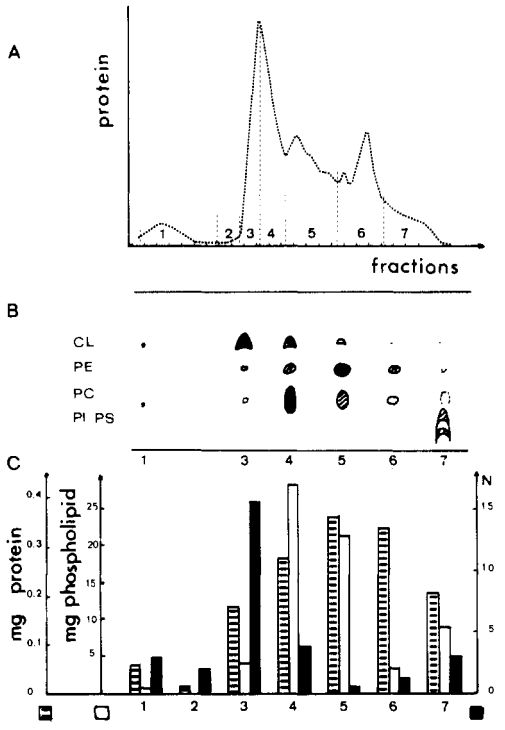


FIGURE 1: Chromatography of a cold chloroform extract of Gal mitochondria (obtained by method II) through a Sephadex LH-20 column, according to Kadenbach & Hadvary (1973b). (A) Typical elution profile obtained with proteins determined by Lowry's method. Proteins are pooled in several fractions (1 to 7). (B) Qualitative phospholipid analysis of each of the pooled fractions by chromatography on silica gel plate (chloroform-methanol-water, 80:25:4). (○ ⊘ ●) Colored according to the intensity. (C) Quantitative analysis of the pooled fractions. (▤) Total protein; (□) total phospholipids; (■) specific binding activity; $N$ = bound nmol of phosphate per mg of protein. [[$^{32}$]Phosphate] = 10 μM. Time of mixing = 1 min.

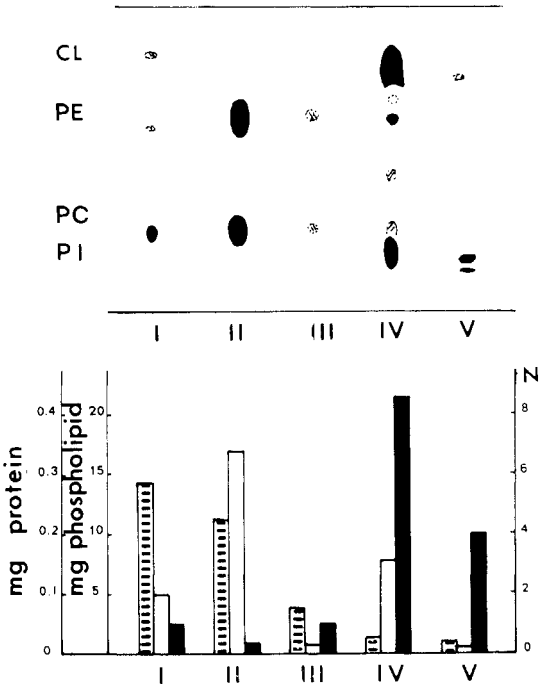


FIGURE 2: Chromatography of a cold chloroform extract (obtained by method II) through a column of DEAE-cellulose acetate, according to Allen et al. (1966). (I) Chloroform-methanol (19:1); (II) chloroform-methanol (9:1); (III) methanol; (IV) 3 g/L of ammonium acetate in chloroform-methanol (2:1); (V) 12 g/L of ammonium acetate in chloroform-methanol (2:1). In each fraction (I to V) qualitative and quantitative analyses of proteins, phospholipids, and binding activity were done as in Figures 1B and 1C.

dylserine (Figure 1B). The phosphate binding activity was measured on each fraction. Only the protein fraction associated with cardiolipin had a high affinity for phosphate (Figure 1C). In the subsequent fractions a low binding activity was found.

Since the main active fraction was associated with cardiolipin, the crude proteolipid purification was performed with another chromatographic procedure based on ion exchange of the polar lipids on DEAE-cellulose acetate, according to Allen et al. (1966) (Figure 2). In this system, phospholipids and the associated proteins are sequentially eluted, first with mixtures of chloroform-methanol and then with chloroform-methanol containing increasing amounts of ammonium acetate. Only the protein fraction very rich in negatively charged phospholipids (cardiolipin and phosphatidylinositol) supported a high $P_i$-binding activity; the specific activity of this fraction was of the same order of magnitude as that of proteolipids purified by Sephadex LH-20 chromatography. Then, the active fraction was chromatographed on silica gel G (chloroform-methanol-water, 80:25:4). The binding activity was found at the cardiolipin level and at the phosphatidylinositol level (not shown), while the phospholipids alone were inactive (see below).

Then, in order to ascertain the role of the cardiolipin in the active fraction, the purified proteolipid was submitted to two-dimensional chromatography on paper impregnated with EDTA according to Steiner & Lester (1972). In this way, all

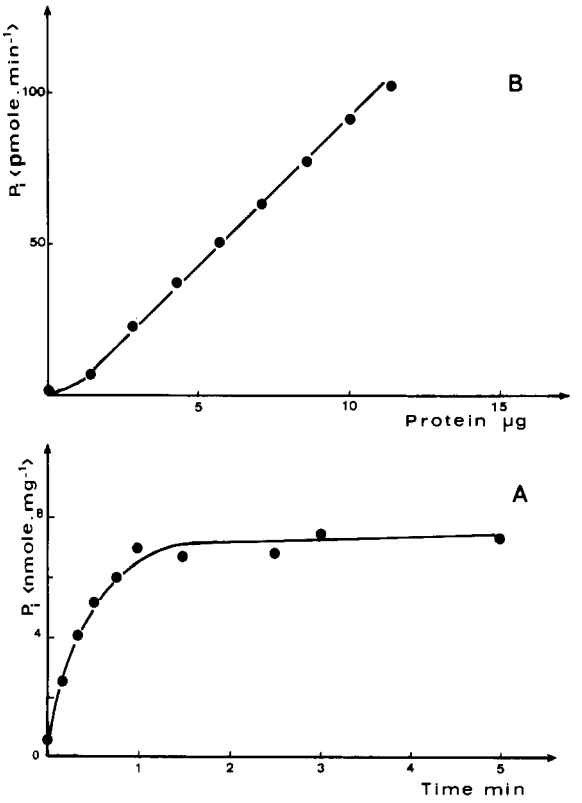


FIGURE 3: Measurement of the phosphate binding activity of the purified proteolipid as a function of: (A) the time of mixing; proteolipid = 4 µg of protein/assay; [[$^{32}$P]phosphate] = 10 µM (see Methods); (B) the protein concentration of the proteolipid; time of mixing = 1 min; [[$^{32}$P]phosphate] = 10 µM.

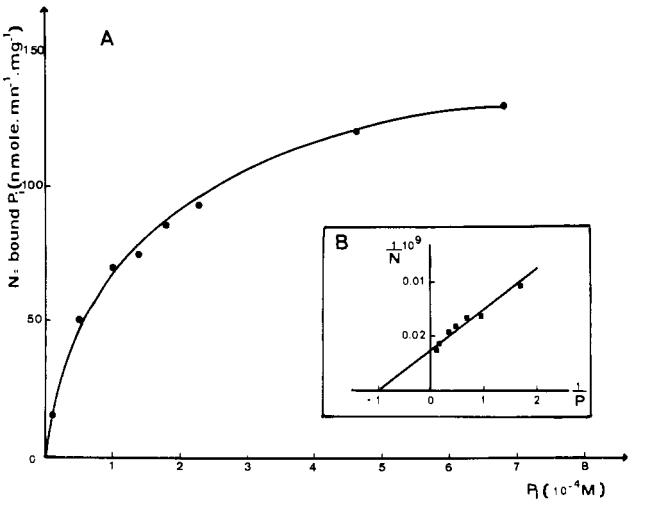


FIGURE 4: (A) Dependence of the phosphate binding on the external phosphate concentration. Proteolipid = 5 µg of protein. Time of mixing = 1 min. (B) Reciprocal plot of Figure 5 (upper curve).

TABLE II: Control of the Validity of the Binding Experiments[a]

| Assay conditions | | dpm |
|---|---|---|
| Changes in the chloroformic phase | Chloroform alone | 160 |
| | +NEM | 200 |
| | +PC, 0.25 mg | 150 |
| | +PC, 1.3 mg | 300 |
| | +CL, 0.25 mg | 400 |
| | +CL, 5 mg | 700 |
| | SAB extract[b] | 180 |
| | Crude proteolipid (8 µg) | 13 000 |
| | Purified proteolipid (fraction 3, Figure 1), 3.5 µg | 45 000 |
| | +NEM | 9 000 |
| | Fraction 5 (Figure 1), 2.5 µg | 2 000 |
| | +NEM | 3 000 |
| | +CL | 2 100 |
| Chloroform washing phase under various conditions | Control | 11 000 |
| | Buffer without $P_i$ | 9 200 |
| | Buffer + cold $P_i$, 1 mM | 6 000 |

[a] Binding test was performed with phosphate 10 µM (specific activity = 8 × $10^5$ dpm/nmol). Mixing time = 1 min. [b] SAB extract: bovine serum albumin was treated by the first method of extraction. Phospholipids: PC = dipalmitoylphosphatidylcholine (Sigma); CL = cardiolipin from beef heart (Sigma). Washing of the chloroform phase was realized by doing first the normal binding test, taking out the aqueous phase, and mixing again the chloroformic phase (1 min) with different buffers.

the phospholipids could be separated. Each spot was cut out, eluted from paper, and submitted to protein determination and to the $P_i$-binding test. The active fraction was found to be associated with cardiolipin and its activity was increased four times (not shown).

Preliminary $NaDodSO_4$ electrophoresis, according to Cattell et al. (1972), in order to determine the molecular weight of the active protein, was difficult, due to the presence of phospholipids. However, it seemed that the chloroform extract contained at least two major bands of protein with molecular weights around 28 000 and 8000. The active fraction purified by Sephadex LH-20 chromatography showed a large band between 8000 and 10 000.

*II. Characterization and Specificity of the Proteolipid.* In such binding experiments as those described above, an artifactual binding could take place. Levinson & Keynes (1972) had shown that in acetylcholine receptors isolated by chloroform–methanol extraction and subsequent purification on Sephadex LH-20 column, an artifactual ligand binding could be observed.

In an attempt to investigate this possibility, several experiments were performed, testing the validity and the stability of the phosphate binding. The validity was controlled by mixing the [$^{32}$P]phosphate buffer with solutions of pure phospholipid or mixtures of one of the inactive fraction with cardiolipin. In all of the cases no increase of the radioactivity was found in the chloroformic phases (Table II). By washing the chloroformic phase after the binding test with a buffer containing or not containing cold phosphate, a certain amount of bound phosphate could be either released or exchanged (Table II).

The phosphate binding activity of the proteolipid purified on Sephadex LH-20 was tested as a function of several parameters. Figure 3A shows the time course of the phosphate incorporation in the chloroformic phase; the maximum incorporation is reached after 1 min of mixing. This incorporation is directly proportional to the protein concentration (Figure 3B) except below 3 µg of protein/mL.

The $P_i$-binding activity, studied as a function of the external phosphate concentration, exhibits a saturation behavior (Figure 4). Although measurements were performed in a heterogeneous medium, an approximate value of the apparent binding constant ($K$), defined according to Pressman (1973) as a two phase association constant, was calculated: $K \simeq 0.1$ mM.

The thiol reagent sensitivity was tested by using mersalyl or NEM, but data obtained with NEM were more reproducible. In these experiments 50 µL of NEM (100 mM) in an aqueous solution, chloroform saturated, was mixed for 3 min

TABLE III: Comparative Activities between the ATPases of Different Origins and the Proteolipids Extracted from Them.

| Type of organelles | Respiratory rate on ethanol + ADP (natom of $O_2$ $mg^{-1}$ $min^{-1}$) | ATPase act.[b] ($\mu$mol of phosphate/(mg of protein/min)) | | | Binding act. of proteolipid from ATPase (nmol of bound $P_i$/mg of protein) | |
|---|---|---|---|---|---|---|
| | | Mitochondria | After gradient | After Sepharose | Crude | Purified |
| Gal mitochondria | 600 | 1.4 (78) | 8.2 (100) | 18 | 0.28 | 5.6 |
| Baker's yeast mitochondria[a] | | 0.9 (85) | 6 (70) | 15 | 0.34 | 6.1 |
| CAP mitochondria | 25 | 0.94 (9) | 7.2 (10) | | 0.1 | Nonsignificant |
| "Petite" mitochondria | | 0.15 (0) | 4 (0) | | 0.01 | Nonsignificant |

[a] Mitochondria were isolated from baker's yeast according to Tzagoloff (1968). [b] % oligomycin sensitivity in parentheses.

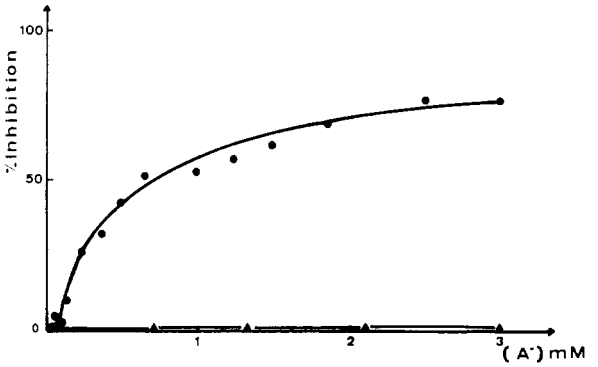


FIGURE 5: Phosphate binding inhibition by another anion: (●—●) arsenate; (▲—▲) succinate.

with the chloroformic solution of proteolipid before the [$^{32}$P]phosphate solution addition.

The NEM sensitivity of the proteolipid appeared after purification by chromatography (Table I); it still reached 75% inhibition of the phosphate binding. The fractions with low $P_i$-binding activity were not inhibited by NEM, but, on the contrary, stimulated (not shown).

Previous work on rat liver mitochondria has shown that arsenate was a competitive inhibitor for phosphate (Guérin et al., 1975). Inhibition by arsenate was therefore tested on the $P_i$-binding activity of the proteolipid. The inhibition curve obtained with increasing concentrations of arsenate is shown in Figure 5: the arsenate concentration required for half inhibition was about 0.8 mM. This inhibition effect of arsenate seems specific since, when similar experiments were performed with other anions (for example, succinate), no variation of the $P_i$-binding activity was found (Figure 5).

*III. Origin and Localization of the Proteolipid.* It is known that several mitochondrial proteins are dependent on mitoribosomal protein synthesis (for a review, see Tzagoloff et al., 1973; Schatz & Mason, 1974). Particularly, we have demonstrated that one of the consequences of chloramphenicol addition to the growth medium of yeast was a mitochondrial phosphate transport modification (Rigoulet et al., 1977). In mitochondria isolated from yeast grown in the presence of chloramphenicol, the phosphate transport did not follow saturation kinetics in contrast to the fully differentiated mitochondria; however, the phosphate penetration was mersalyl-sensitive. Similar results were obtained with mitochondria isolated from a mutant "petite colonie" of this strain, mitochondrial DNA deficient. Therefore, proteolipids were extracted from mitochondria isolated from yeast grown in the presence of chloramphenicol and from mutant "petite colonie".

Both types of organelles were tested in their respiratory capacity and in their oligomycin-sensitive ATPase activity. CAP mitochondria were never fully repressed since a residual oligomycin sensitivity was found; mitochondria from "petite" mutant were fully repressed (Table III).

Both extraction methods were used to obtain the chloroform extract. As shown in Table I, crude proteolipids extracted from CAP mitochondria or from "petite" mitochondria had a lower binding activity than that extracted from Gal mitochondria, while the ratio mg of phospholipid/mg of protein was higher. After purification, the proteolipid from CAP mitochondria represented only 1% of the total eluted protein, while in Gal mitochondria the active fraction represented about 12%. However, this remaining fraction kept a specific phosphate binding activity quite similar to the specific activity of the proteolipid from Gal mitochondria (Table I). At the same time, it was NEM sensitive. In contrast, when the crude proteolipid from the mutant "petite colonie" was purified, no active fraction was found.

The mitochondrial proteins dependent on mitoribosomal protein synthesis are essentially localized in the "membrane factor" of the oligomycin sensitive ATPase and in the cytochrome oxidase (for review, see Tzagoloff, 1975; Schatz & Mason, 1974). Preliminary experiments aimed to search for the occurrence of the proteolipid in the ATPase of different types of mitochondria.

ATPases were isolated and purified according to Ryrie (1976), except that the Triton extract, after concentration in a Hollow-fiber beaker, was purified on a discontinuous gradient of glycerol: 6, 12, 18, 24, 48% of glycerol in the same buffer as Ryrie (1976). ATPase was collected in the 18% glycerol phase. After concentration the enzyme was purified through a column of Sepharose 4B, according to Ryrie (1976). Purity of the preparations was controlled by electrophoresis (Figure 6). In Table III are summarized the different steps of the ATPase purification and the characteristics of the proteolipid extracted from these enzymes.

First OS-ATPases were purified from Gal mitochondria and from mitochondria isolated from Bakers' yeast according to Tzagoloff (1968). Both ATPases have the same characteristics. Proteolipids were extracted from these enzymes and tested in the phosphate binding activity. It can be seen (Table III) that the specific activity of this proteolipid was of the same order of magnitude as that extracted from the mitochondria. Also it was NEM sensitive, and its activity was inhibited by arsenate. The protein fraction was associated with cardiolipin and also with phosphatidylinositol.

From ATPase from CAP mitochondria, a very small amount of proteolipid was extracted, having little affinity for phosphate. The ATPase of the mutant "petite colonie" was isolated and purified as the OS-ATPase. The electrophoretic patterns of the ATPases isolated from Gal mitochondria and from "petite" mitochondria were compared in Figure 6. In the ATPase from "petite" mitochondria, some bands are lacking,

essentially the 45 000 band which is attributed to a membrane factor unit synthesized on the mitoribosomes (Tzagoloff & Akai, 1972). From the ATPase of the mutant "petite colonie" the proteolipidic fraction isolated was without activity. However, all the phospholipids were present.

## Discussion

It has been shown by Kadenbach & Hadvary (1973b) that a chloroform extract from rat liver mitochondria was able to bind specifically phosphate. The proteolipid responsible for this binding could be partially separated from the other components of the extract and was proposed by these authors as a good candidate for the phosphate carrier.

This paper tries to answer three questions related to the involvement of a mitochondrial proteolipid in phosphate transport.

(1) Is there a proteolipid able to bind phosphate in yeast mitochondria and, in such a case, what are its characteristics?

(2) What are its origin and its localization?

(3) Is it a part of the phosphate carrier?

Experiments described in this paper show that such a chloroform soluble protein can be extracted from yeast mitochondria. Purification procedures and further characterization of this peculiar protein are described.

The main point lies in the importance of the phospholipids. The chloroform extract always contains phospholipids, but its activity in phosphate binding is proportional to the amount of phospholipids linked to the protein: two extractions procedures were compared (Table I). The first, according to Kadenbach & Hadvary, uses a partial solubilization of the lipidic compounds after a $Cl_3CCOOH$ precipitation of proteins. The second is carried out in such a way as to allow a complete phospholipid extraction (Method II): it can be seen that the $P_i$-binding activity of the proteolipid in such a case is increased by a factor 5 and the ratio phospholipid (mg)/protein (mg) is at the same time the highest, pointing out the importance of the presence of these lipids. Indeed, the active fraction is always associated with negatively charged phospholipids, essentially cardiolipin.

It is quite surprising that a protein of such a molecular weight, estimated to be about 8000, could be separated by the Sephadex LH-20 stationary phase, the exclusion limit of which is about 2 to 4000. In fact Sephadex LH-20 separation is not a filtration chromatography of proteins but rather a partition chromatography in which proteins are eluted in association with phospholipids, according to the affinity of these complexes for each phase. This conclusion is corroborated by the proteolipid purification either by ion-exchange or by thin-layer chromatography of lipids. Data presented in this paper show that in each case the chloroform soluble proteins are not separated according to their specific properties but according to the properties of the molecular species of the associated phospholipid.

As far as the proteolipid is concerned, it must be stressed that yeast represents a very suitable material since it is possible to obtain modified mitochondria either by the addition of a mitoribosomal protein synthesis inhibitor (chloramphenicol) to the growth medium or by the utilization of a mitochondrial DNA mutant. From previous work in the laboratory it was shown that the phosphate transport system is dependent on mitochondrial protein synthesis (Guérin et al., 1977; Rigoulet et al., 1977). For instance, the phosphate exchange in CAP mitochondria or in mitochondria from "petite mutant" does not follow a saturation kinetics in the tested range of the external phosphate concentration (0–10 mM), although the phosphate uptake, in this case, is inhibited by mersalyl. These organelles are swelled in isoosmotic phosphate solutions as are differentiated mitochondria (Arselin de Chateaubodeau et al., 1976). From these results we have proposed that the phosphate transport system is composed of, at least, two components: the first component, alone, is sufficient to permit a mersalyl-sensitive phosphate uptake but its affinity for phosphate is rather low; a second component is required for the complete phosphate transport system defined by the saturation kinetics. Probably this component binds phosphate with a good affinity, and its synthesis or its integration in the membrane depends on the mitoribosomal protein synthesis.

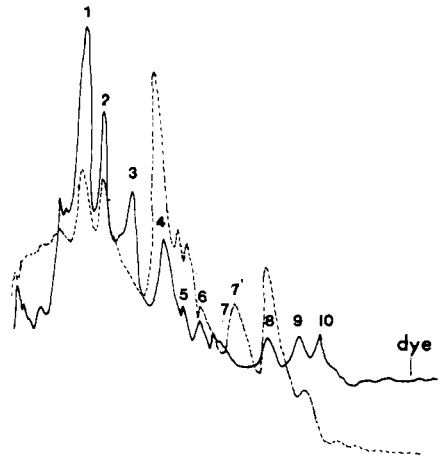


FIGURE 6: $NaDodSO_4$ electrophoresis of the ATPases of Gal mitochondria and of "petite" mitochondria on 10% polyacrylamide gel slab, according to Weber & Osborn (1969). Staining in Coomassie blue was done over 15 h. After destaining, scanning was performed with a photometer integrator Vernon. Molecular weights of the bands were: (1) 58 800; (2) 53 000; (3) 44 700; (4) 38 000; (5) 31 000; (6) 27 000; (7) 24 500; (7') ("petite" mitochondria) 22 000; (8) 17 500; (9) 12 000; (10) 10 900.

Attempts have been carried out in some laboratories in order to isolate the $P_i$ carrier from mammalian mitochondria. A specific labeling by radioactive *N*-ethylmaleimide of a protein was obtained (Coty & Pedersen, 1975; Hadvary & Kadenbach, 1976). For that experiment, SH groups of the $P_i$ carrier were protected by a mercurial and then mitochondria treated by an excess of unlabeled NEM. After washing in order to eliminate mercurial, mitochondria were labeled with radioactive NEM. A direct labeling with [$^{14}$C]mersalyl has been also reported (Briand et al., 1976). Therefore, in these three reports the isolated proteins seem to concern the first component of the transport system, i.e., that conferring the sensitivity of the $P_i$-transport toward mersalyl.

Some data described in this paper suggest that the proteolipid could be the second component of the $P_i$ carrier. The $P_i$-binding activity of the proteolipid can be characterized by several properties: saturation effect by ligand; inhibition by a substrate analogue arsenate. Also in CAP mitochondria, the amount of $P_i$-binding protein is dramatically decreased, and in "petite" mitochondria the proteolipid has quite disappeared.

A proteolipid can be extracted from purified OS-ATPase which binds phosphate. It has similar activity and properties as the proteolipid extracted from whole mitochondria. Its activity is also correlated with the presence of associated cardiolipin.

All these results led to the question of the structural relationship between the $P_i$ carrier and the OS-ATPase complex. With a different experimental approach we were able to show

that a saturation kinetics of the phosphate transport was detected only with mitochondria supporting an oligomycin-sensitive ATPase activity. More accurately, the biogenesis of the phosphate carrier parallels the biogenesis of the OS-ATPase complex (Rigoulet et al., 1977).

It is not known at this time whether these mitochondrial translation products are structural or catalytic components of the inner membrane complexes. The highly hydrophobic protein purified during this work seems to be a good candidate for acting as a "carrier" in the $P_i$ transport across the membrane lipidic phase. This hypothesis can be related to the recent work of Folch-Pi & Sakura (1976) who have isolated from different material and from mitochondria a proteolipid associated again with phospholipids. When this protein was delipidated, it became water soluble. This change in solubility was correlated to a structural modification of this protein ($\alpha$-helix content change). These results may help the understanding of the transport process across a membrane.

Further experiments are under way to elucidate the interaction between phospholipids and the protein to gain better understanding in the $P_i$ transport process.

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