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EFFECT OF PHOPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE ENRICHMENT ON THE STRUCTURE AND FUNCTION OF YEAST MEMBRANE

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The phospholipid composition of yeast plasma membrane was manipulated by two different methods: (i) by using two auxotrophic strains KA101 (cho1) and MC13 (Cho^+) which required phospholipid bases for growth and (ii) by supplementing *Saccharomyces cerevisiae* (3059) cells with high concentration of choline or ethanolamine. It was possible to enrich the plasma membrane with phosphatidylcholine (PC) or phosphatidylethanolamine (PE) by both methods. The uptake of amino acids, e.g., glycine, glutamic acid, leucine, lysine methionine, phenylalanine, proline and serine, was significantly reduced in PC- or PE-enriched cells. However, the extent of reduction in transport was variable among different strains. A fluorescent probe, 1-anilino-8-naphthalene sulfonate (ANS), was used to monitor the structural changes induced by altered phospholipid composition. It was observed that the relative fluorescence intensity of bound ANS was decreased as a consequence of PC or PE enrichment. The decrease in fluorescence was probably associated with reduced number of available binding sites (n) and increased apparent dissociation constant (K_d). Furthermore, our results also suggest that a critical level of PE or PC is required for proper functioning of yeast membrane.

Introduction

The lipid composition of yeast plasma membrane can be manipulated in a predictable manner by varying the nature and composition of the environment [1–5]. The same can also be achieved by using mutants unable to synthesize specific membrane lipids [6–8]. Similar to various prokaryotic and eukaryotic systems [9–15], the altered lipid composition of yeast may help in elucidating the physiological role of lipids. The transport phenomenon in yeast is well characterized [16–24]; however, the role of lipids in its cellular permeability is less documented.

Our earlier studies demonstrated that the lipid composition was significantly altered in *Candida albicans* cells, grown in the alkanes of varying chain length [25]. In such cells different carrier(s), responsible for the uptake of several amino acids, behaved differently towards the altered lipid composition. Since the change in lipids of alkane-grown cells was of general nature, no specific role of a lipid component could be envisaged in that study. In the present study, we have used two auxotrophs of choline and ethanolamine which accumulated PC or PE, respectively. The supplementation of *Saccharomyces cerevisiae* (3059) with high concentration of choline or ethanolamine also resulted in PC or PE accumulation. The effect of such

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; ANS, 1-anilino-8-naphthalene sulfonate; PS, phosphatidylserine.

phospholipid changes on amino acid transport is investigated. The structural alteration as a result of PC or PE enrichment in yeast membrane is ascertained by using ANS as a fluorescent probe.

Materials and Methods

Materials. Ethanolamine, choline chloride and standard phospholipids were purchased from Sigma Chemical Co., U.S.A. Various L-[^{14}C]amino acids were from Bhabha Atomic Research Centre, Bombay, India. ANS (magnesium salt) was procured from Serva Fine Biochemicals, Heidelberg, F.R.G. Other chemicals used were of analytical grade.

Maintenance of strains and growth conditions. The parental strain of *Saccharomyces cerevisiae* (ade5α) and mutant strains KA101 (αcho1, ino1, lys2, can1) and MC13 (αCho$^+$, ino1, lys2, can1), requiring choline or ethanolamine for growth, were obtained from Professor Susan A. Henry, Albert Einstein College of Medicine, Bronx, New York, U.S.A. The mutant strains were earlier designated as cho1 and Cho$^+$ and their properties and synthetic media suitable for their growth were described by Atkinson et al. [6]. These mutant strains had shown high frequency of reversal and the colonies were, therefore, replicated from time to time from YEPD (2% glucose, 2% peptone, 1% Difco yeast extract) to synthetic minimal medium supplemented with choline (1 mM) or ethanolamine (1 mM). In all the experiments, these strains were first grown in YEPD and transferred to synthetic minimal media with 1 mM ethanolamine or 1 mM choline, or with no supplement, at 30°C.

Another strain, *S. cerevisiae* (3059), was obtained from the National Chemical Laboratory, Pune, India. The culture was in a complex nutrient medium and cultivated in defined mineral salt solution containing 0.3% (w/v) KH_2PO_4, 0.3% (w/v) $(NH_4)_2SO_4$, 0.025% (w/v) $CaCl_2$, 0.025% (w/v) $MgSO_4$, 0.001% (w/v) biotin with the addition of carbon source of 0.5% (w/v) glucose, and supplemented with 20 mM choline, 20 mM ethanolamine, or not at all.

Cells of exponential growth phase were harvested by centrifugation at $1500 \times g$ for 10 min and washed thrice with sterile distilled water before use in the various studies.

Preparation of crude plasma membrane fraction. Spheroplasts, prepared following the method of Jayakumar et al. [26], were suspended in 0.6 M sorbitol and ground with glass beads (0.45–0.52 mm) for 1 h in the cold (0–4°C). However, due to incomplete disruption, the suspension was sonicated at a power of peak-to-peak amplitude 12 μm by using a 150 W ultrasonic disintegrator MK_2 (MSE) for 1 min at 2–4°C. Lysed suspension was differentially centrifuged at $5000 \times g$ for 10 min and the resulting supernatant was centrifuged at $10000 \times g$ for 30 min. This was done to remove cell debris and mitochondrial fractions, respectively. The supernatant obtained after removing mitochondrial fraction was further centrifuged at $105000 \times g$ for 1 h. The pellet thus obtained has been designated as crude plasma membrane fraction.

Lipid extraction and analysis. The method of Folch et al. [27] was used for total lipid extraction. Phospholipids were separated by thin-layer chromatography using Silica Gel-G as an adsorbant [25,28]. Protein was determined by the method of Lowry et al. [29], using bovine serum albumin as a standard.

Transport assay. Transport assay procedure was essentially similar as described earlier [25]. The reaction mixture containing cells (150–200 μg protein/ml) was preincubated at 30°C for 10 min with cycloheximide (200 μg/ml) to inhibit protein synthesis. The reaction was initiated by the addition of L-[^{14}C]amino acids (1.66 mM lysine, 1 mM proline, 0.83 mM glutamic acid, 0.55 mM glycine, 2 mM phenylalanine, 2 mM leucine, 1.5 mM methionine and 1.5 mM serine) to the assay mixture. The amino acid concentrations were 2–3 times higher than their respective K_m values of cells without supplemented media. After 10 min incubation, the reaction mixture was diluted 50-fold in chilled water. The diluted suspension was rapidly filtered through 0.45 μm Millipore membrane filter discs. The radioactivity retained on filter discs was determined in a Packard scintillation counter using a toluene-based scintillation fluid.

Measurement of fluorescence. The fluorescence of ANS, associated with various plasma membranes, was measured in a spectrofluorimeter assembled in the laboratory [30]. The excitation

wavelength (396 nm) was obtained through a monochromator with a tungsten lamp as the excitation source. The front surface emission was measured at 460 nm through another monochromator at 90° to the direction of exciting light. The fluorescence spectra were corrected for scattering and inner filter effect [31]. The number of binding sites (n) of ANS bound to various membranes was obtained from Scatchard plots [32].

Results and Discussion

Phospholipid composition

The two auxotrophic mutants, chol and Cho^+, have already been well characterized [33]. Chol mutant appears to use almost exclusively the alternative pathway described by Kennedy and Weiss [34] for the production of PE and PC, bypassing phosphatidylserine (PS) as an intermediate. However, the activity of methyltransferase in the auxotroph Cho^+ is probably altered [33]. These mutant cells were unable to grow in medium deficient in phospholipid bases but remained viable for some time. Due to the difference in phospholipid synthetic pathway of these mutants, the phospholipid composition of non-supplemented cells was different. For example, the levels of PC and PE were very low in chol due to non-supplementation of exogenous bases. Therefore, in the present work the phospholipid composition and amino acid transport in choline- or ethanolamine-grown cells has been compared with their parent wild-type strain (ade5α).

The phospholipid composition of wild-type, chol and Cho^+ strains was examined under different growth conditions. Crude plasma membrane fraction, isolated from different cells, was used for phospholipid extraction and analysis. As can be seen from Table I, both chol and Cho^+ when grown in media containing choline chloride (1 mM) resulted in PC accumulation. As compared to the wild-type strain, the increase in PC content was about 15% in both the auxotrophs. However, when these auxotrophs were grown in media containing ethanolamine (1 mM), the increase in total PE content was between 30 and 55%. The significant decrease in PC levels of Cho^+ cells grown in media containing ethanolamine is probably due to its defective methyltransferase(s) activity [33]. It is pertinent to mention that the addition of either choline or ethanolamine to the growth media of wild-type strain did not change the levels of PC or PE [33].

In addition to the auxotrophic strains, we have also used wild-type strain, *S. cerevisiae* (3059), cells grown in 20 mM choline- and 20 mM ethanolamine-supplemented media [35]. This was done to ascertain if the percentage increase in PC

TABLE I

PHOSPHOLIPID COMPOSITION OF CRUDE PLASMA MEMBRANE OF *SACCHAROMYCES CEREVISIAE*

Determination of lipid phosphorus and identification of different phospholipids were done as described under Materials and Methods. Phosphorus contents were multiplied by 25 to give the total phospholipid contents. All the values are an average of three to five separate determinations.

Strain	Medium supplemented	Total phospholipid (%)				
		PI + PS	PC	PE	CL	PC/PE [a]
ade5α	none	23.9	48.5	19.1	8.5	2.6
KA101 } (chol)	choline	25.8 [b]	56.5	14.5	3.3	3.9
KA101 } (chol)	ethanolamine	26.6 [b]	44.7	24.7	4.0	1.8
MC13 } (Cho^+)	choline	24.8	55.5	12.6	7.1	4.4
MC13 } (Cho^+)	ethanolamine	24.9	36.2	29.8	9.1	1.3
3059	none	20.6	42.9	16.7	4.2	2.6
3059	choline	20.0	60.0	14.0	3.0	4.3
3059	ethanolamine	20.0	37.5	25.0	7.5	1.5

[a] Ratio

[b] The strain has been shown to lack any detectable phosphatidylserine [33].

and PE contents obtained by two different methods would have a similar effect on yeast membrane. Table I demonstrates that there was about 40–50% increase in PC and PE contents in cells grown on choline- or ethanolamine-supplemented media, respectively. As compared to both the auxotrophic strains, the percentage increase in PC content was more in choline-supplemented cells (Table I). The contents of other phospholipids were not much affected except in the case of chol membranes, where cardiolipin was 50–60% less as compared to wild-type. It became evident from both sets of studies that the phospholipid composition of yeast plasma membrane can be influenced predictably by manipulating the growth conditions.

Amino acid transport in choline and ethanolamine auxotrophs

Table II shows the effect of PC and PE increase on the transport of phenylalanine, methionine, serine, leucine, glycine, lysine, glutamic acid and proline. The observed reduction in the accumulation of all amino acids was between 12% and 70% in both choline- and ethanolamine-grown cells of chol. In general the pattern was the same when Cho^+ cells were grown in choline- or ethanolamine-containing medium where the reduction in the uptake of amino acids was between 10% and 40% (Table II). The kinetic data revealed that the apparent K_m values of all the amino acids in chol and Cho^+ auxotrophs grown in choline or ethanolamine were increased as compared to wild-type. This indicates a decrease in the affinities of the carrier(s) as a consequence of PC or PE enrichment (Table III). The V_{max} values, however, were not much affected in these cells (data not shown). The addition of choline or ethanolamine to wild-type (ade5α) cells neither changed PC or PE contents [33] nor had any effect on amino acid transport.

It is apparent that the extent of reduction in the total accumulation of glycine, leucine, serine and proline was significantly different among the two auxotrophic strains. These differences can be attributed to many factors, e.g., the PC/PE ratio, the composition and the positional distribution of fatty acids of these phospholipids [36,37]. However, the difference in serine uptake among the two strains needs special mention. The chol mutant completely lacks PS, since the mutant is deficient in its synthesis [33]. The existence of an alternate biosynthetic pathway utilizing free ethanolamine or choline permits yeast to circumvent the requirement for PS as a precursor for other lipids [34]. As can be seen from Table II, the transport of serine was reduced upto 70% in both choline- and ethanolamine-grown chol cells. In contrast, the serine uptake was affected only 30–40% in Cho^+ cells. The difference among the two strains could probably be due to the fact that PS requirement is dispensable for chol cells, and therefore the trans-

TABLE II

AMINO ACID UPTAKE IN *SACCHAROMYCES CEREVISIAE*

Uptake of various amino acids in various cells was followed as described in Materials and Methods. The changes observed in uptake of amino acids are statistically significant, since P values range from <0.001 to <0.05.

Strain	Medium supplemented	Amino acid uptake (μmol/mg protein per 10 min)							
		Glu	Gly	Leu	Lys	Met	Phe	Pro	Ser
ade5α	none	0.28	0.83	1.06	1.01	0.31	0.55	0.24	0.10
KA101 } (chol)	choline	0.24	0.34	0.62	0.78	0.16	0.41	0.12	0.03
KA101 } (chol)	ethanolamine	0.15	0.30	0.55	0.72	0.19	0.40	0.10	0.02
MC13 } (Cho^+)	choline	0.28	0.43	0.66	0.87	0.21	0.47	0.21	0.07
MC13 } (Cho^+)	ethanolamine	0.27	0.49	0.76	0.91	0.22	0.47	0.23	0.06
3059	none	0.75	0.18	0.49	0.96	0.30	0.61	0.22	0.28
3059	choline	0.71	0.16	0.34	0.53	0.29	0.43	0.14	0.25
3059	ethanolamine	0.62	0.12	0.40	0.27	0.15	0.47	0.12	0.10

TABLE III

K_m VALUES OF AMINO ACIDS IN *SACCHAROMYCES CEREVISIAE*

Various concentrations (0.05–0.5 mM) of amino acids were prepared with the same amount of radioactivity. All the assay conditions were same as described for transport studies with the exception of different concentrations of amino acids. For the determination of K_m, the reaction was terminated after 30 s. K_m values are an average of two or three separate determinations. The changes observed in K_m are statistically significant since P values range from <0.01 to <0.05.

Strain	Medium supplemented	K_m (mM)							
		Glu	Gly	Leu	Lys	Met	Phe	Pro	Ser
ade5α	none	0.38	0.25	0.30	0.60	0.59	0.30	0.10	0.71
KA101 } (chol)	choline	0.44	0.43	0.75	0.83	0.83	0.63	0.22	2.00
KA101 } (chol)	ethanolamine	0.55	0.50	0.83	1.00	0.77	0.83	0.30	2.00
MC13 } (Cho^+)	choline	0.45	0.37	0.50	0.78	0.75	0.50	0.15	1.00
MC13 } (Cho^+)	ethanolamine	0.50	0.45	0.75	0.78	0.60	0.75	0.15	1.50
3059	none	0.22	0.33	0.25	0.44	0.30	0.28	0.08	0.10
3059	choline	0.25	0.38	0.50	0.59	0.32	0.34	0.16	0.15
3059	ethanolamine	0.25	0.50	0.43	0.83	0.59	0.30	0.21	0.27

port capacity, which may be related to its utilization, is also reduced significantly. However, whether the transport of serine is linked to its utilization as phospholipid precursor cannot be answered from these results. The differences in the accumulation of other amino acids in the two auxotrophic strains could also be due to the difference in PS contents.

Amino acid transport in choline- and ethanolamine-supplemented cells

When *S. cerevisiae* (3059) cells were grown in presence of 20 mM choline or 20 mM ethanolamine-supplemented medium, their membrane phospholipids became enriched with PC or PE, respectively (Table I). It is known that 10–20 mM concentrations of choline or ethanolamine give maximum enrichment of PC or PE, respectively [35]. As compared to glucose-grown cells, PC and PE enrichment resulted in the reduction of transport of all the eight amino acids (Table II). The extent of reduction in amino acid transport between auxotrophic strains and supplemented cells was significantly variable. For instance, the reduction in the uptake of lysine was 45–70% in choline- or ethanolamine-supplemented cells and was only 10–18% in auxotrophic strains. On the other hand, the reduction in glycine accumulation in auxotrophic strains was more pronounced as compared to the supplemented cells (Table II). However, similar to auxotrophic strains, the reduction in the uptake was associated with increased K_m values (Table III). It would mean that a critical concentration or level of each phospholipid is a prerequisite for proper functioning of the plasma membrane. In several bacterial species, reports are available to demonstrate that the transport of various nutrients are significantly affected due to altered phospholipid composition [38–41]. From such studies, it is possible to explore the precise role of membrane phospholipids in yeast membrane functions.

ANS response in PC- or PE-enriched spheroplasts

The conformational changes due to PC or PE enrichment in membrane were monitored by using a fluorescent dye, ANS [42–46]. It was observed that ANS did not give a detectable response when intact cells of various strains were used. Therefore, the fluorescence of ANS was monitored in the spheroplasts of these cells where fluorescence was detectable and reproducible. The lack of response of ANS dye in intact cells may be due to the presence of cell wall which may affect the permeability and binding of the dye.

There was axxxx decrease in the relative fluorescence intensities of bound ANS in both choline- and ethanolamine-grown auxotrophs. However, the

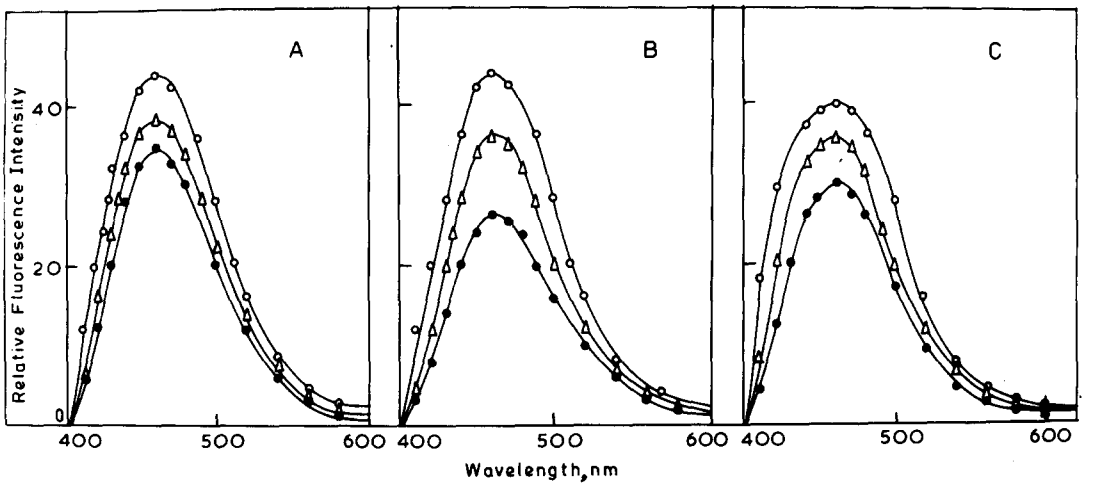

Fig. 1. Emission spectra of ANS. The reaction mixture contained 50 mM Tris-acetate buffer (pH 7.2), 33.3 μM ANS and the concentration of spheroplast protein was 100 μg/ml in each case. Other details were as described under Materials and Methods. A: Spheroplast of wild-type ((O——O); of chol grown in (△——△) choline- and in (●——●) ethanolamine- containing media. B: Spheroplast of wild-type (O——O); Cho^+ grown in (△——△) choline- and in (●——●) ethanolamine-containing media. C: *Saccharomyces cerevisiae* (3059): spheroplast of (O——O) unsupplemented; (△——△) choline- and (●——●) ethanolamine-supplemented media.

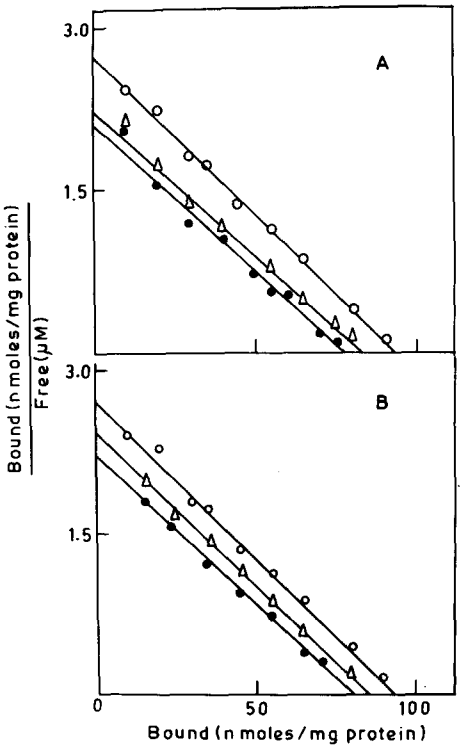

Fig. 2. Scatchard plots of ANS binding to spheroplasts of: (A) wild-type (O——O); chol grown in (△——△) choline- and in (●——●) ethanolamine-containing media; (B) wild-type (O——O); Cho^+ grown in (△——△) choline-; and in (●——●) ethanolamine-containing media. The reaction mixture was same as that of Fig. 1.

decrease in fluorescence was more pronounced (~ 40%) in ethanolamine-grown auxotrophs (Fig. 1A and B). Similar pattern was observed when ANS fluorescence was measured in spheroplasts of choline- or ethanolamine-supplemented cells (Fig. 1C). While analyzing the fluorescence data, it was noted that the phospholipid composition of various strains also revealed a higher percentage increase in PE level (30–35%) in ethanolamine-grown

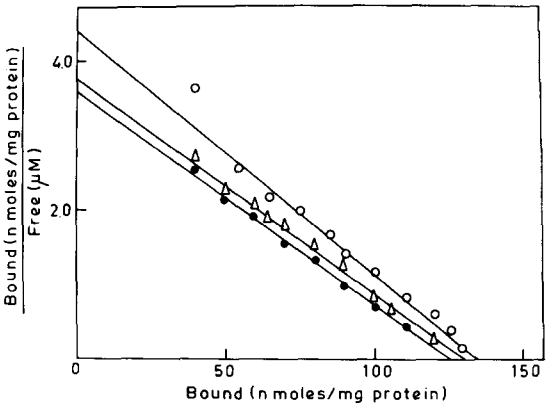

Fig. 3. Scatchard plots of ANS binding to spheroplasts of *Saccharomyces cerevisiae* (3059) : (O——O) unsupplemented; (△——△) choline- and (●——●) ethanolamine-supplemented media.

auxotrophs and in ethanolamine-supplemented cells (Table I). The difference in PE vs. PC levels is reflected in the relative fluorescence of ANS which could be correlated directly to the phospholipid composition. The decrease in relative fluorescence intensity was more pronounced in PE-enriched membranes obtained either by growing chol or Cho^+ in ethanolamine or by supplementing the *S. cerevisiae* (3059) cells with ethanolamine. Under physiological pH, PE is slightly negative charged as compared to PC which is neutral and exists as zwitterion [7]. Therefore, the binding of ANS, which itself is anionic at pH 7, would be decreased in membranes having more PE. It was observed that number of binding sites for ANS were decreased from 105 to 81–77 nmol ANS/mg protein in auxotrophic strains grown in presence of ethanolamine (Fig. 2A and B). Similarly, the binding sites of ANS were reduced in ethanolamine-supplemented cells (Fig. 3). The decrease in ANS fluorescence in PC- or PE-enriched membrane may also partly be due to the changed hydrophobic environment around the embedded dye.

It may be concluded that PC or PE enrichment of yeast plasma membrane results in the alteration of both structure and function. It provides a very useful model system for study of the role of phospholipids in the structure and function of membranes.

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