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Regulation of trehalose metabolism by protein methylation in a mutant strain of *Saccharomyces cerevisiae*

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Abstract

Trehalose is a non reducing disaccharide occurring in a wide range of organisms, from bacteria, yeast, to lower and higher plants and insects. It is economically important as a potent stress protectant, protein and biological membrane stabilizer; hence biosynthesis of trehalose is an extremely important event. Regulation of trehalose metabolism by protein methylation has been reported from previous works of this laboratory. Trehalose metabolism was monitored during different stages of growth of *Saccharomyces cerevisiae*. HPLC and enzymatic determination of trehalose, glucose, trehalose 6 phosphate phosphatase (TPP) and trehalose 6 phosphate synthase (TPS) were carried out. It was noticed that trehalose, glucose, TPP and TPS peaked at A_{660} ~20 during growth but during this period the hydrolyzing enzymes, acid trehalase (AT) and neutral trehalase (NT) are found to be low. Effect of a potent universal methyl group donor S-adenosyl-L-methionine (AdoMet), and a methylation inhibitor, oxidized adenosine (Adox) on trehalose metabolism has been studied. Trehalose metabolism is altered when YPD grown cells were incubated for 1 hr in presence of either 1 mM Adox or AdoMet at 30°C and pH 6.0 TPS showed a slight increase in specific activity in cells incubated with AdoMet over Adox. Trehalose level of Adox treated cells were seen to be lower than control throughout the period of incubation. Trehalose level initially decreased upto 4 hours in all the sets by utilizing pre-synthesized trehalose, thereafter the AdoMet incubated cells showed sharp increase in trehalose content, in 8 hours 3 fold, in 24 hours nearly 5 fold with respect to others. At the point of 48 hours, cells reached stationary phase in all sets and trehalose level was found to increase.

Keywords: S-adenosyl-L-methionine (AdoMet), Acid Trehalase (AT), Oxidized adenosine (Adox), Neutral Trehalase (NT), Trehalose-6-phosphate synthase (TPS), Trehalose-6-phosphate phosphatase (TPP), Trehalose.

Trehalose, commonly known as "insect sugar", is a major storage carbohydrate and is found to be present in a wide variety of microorganisms, plants, and invertebrates except mammals. Accumulation of trehalose in organisms plays a role in enhancing the stress tolerance. This sugar was first isolated from the ergot of rye, in 1832 by Wiggers. Chemically this sugar is α-D-glucopyranosyl- α-D-glucopyranoside. Two glucose molecules are linked together in a α, α 1, 1-glycosidic linkage; hence, trehalose has no reducing power. Due to the absence of reducing ends in the chemical structure, trehalose is highly resistant to heat, pH and Millard's reaction. It accumulates intracellularly during periods of starvation, desiccation and after exposure to mild heat stress, where it serves as a potent membrane stabilizer. Besides stabilizing different cellular structures from adverse effects of freezing and drying induced dehydration and stabilization of proteins against denaturation, trehalose is also important for the control of glucose influx during the cellular response to adverse condition. Trehalose is a multifunctional molecule (Elbein et al, 2003) and the importance of this sugar lies in both economic and biotechnological fields. It is 45 % as sweet as sugar, highly soluble in both water and aqueous ethanol and comparatively less hygroscopic. Industrially it is used for the preservation of color and taste of food products and for protecting vaccines in hot climates. Human red blood cells can be freeze-dried while maintaining a high degree of viability by loading trehalose into them.

Trehalose biosynthesis is controlled by the activity of two synthesizing enzymes, TPS and TPP and two hydrolyzing enzymes AT and NT (Basu et al, 2006; Londesborough and Varimo 1984). Our aim was to find out the regulation of trehalose biosynthesis by post-translational modification of trehalose biosynthesizing enzymes. Protein methylation is one of the modes of protein modification regulating protein function (Aletta et al, 1998). Using AdoMet as the methyl group donor and Adox as the methylation inhibitor, the effect of protein methylation and methylation inhibition on overall trehalose metabolism is being studied.

Materials and Methods

Materials

Trehalose-6-phosphate, Uridine 5'-diphosphoglucose (UDPG), glucose-6-phosphate (G-6-P), Periodate

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Oxidized adenosine (AdOx), S-adenosyl-L-methionine (AdoMet), phenylmethylsulphonyl fluoride (PMSF), benzamidine hydrochloride, 2-mercaptoethanol, Bradford reagent were purchased from Sigma, USA. All the other chemicals and medium components used were of analytical grade and were purchased locally.

Methods

Organism and culture condition

The Kanamycin resistant mutant diploid strain of yeast, *Saccharomyces cerevisiae* MAT aKnu was used. Yeast cells were allowed to grow in YPD medium at 30 ± 1°C shaker incubator till desired growth phase is obtained, which is measured by taking absorbance at 660nm. Cells were harvested by centrifugation at 10 000×g, at 4–5°C for 10min.

Cell lysis and preparation of enzyme solution

Harvested cells were suspended in lysis buffer and cells were disrupted by passing twice through a FRENCH® Pressure Cell Press (SLM Instruments, USA) at 18,000 lb psi. The homogenate was then centrifuged at 10,000 × g, 4 -5 °C for 10 min and the supernatant was dialyzed against 1 l of dialysis buffer with 4- 5 changes following the method of Chaudhuri et al., 2007. Precipitate which appeared after dialysis was removed by centrifugation at 10,000 × g for 10 min. The resulting supernatant was used as crude enzyme solution for different enzyme assays. It was also used to measure protein for determining specific activity of the enzymes.

Assay of different enzymes of trehalose biosynthesis pathway

Trehalose-6-phosphate phosphatase (TPP) was measured according to assay protocol of Matula M., Mitchell M., and Elbein A.D., 1971. Trehalose-6-phosphate synthase (TPS), Acid Trehalase (AT) and Neutral Trehalase (NT) activities were measured according to published protocol of Chaudhuri et al., 2008; Biswas and Ghosh, 1996 respectively.

Trehalose-6-phosphate (1mM) was used as substrate for TPP assay, incubated with $MgCl_2$ at 37°C for 30 min. Phosphate group liberated was quantified spectrophotometrically at A_{530} nm. TPS activity was assayed at 37 °C for 15 min using 5 mM UDPG and 5 mM G-6-P as substrates. Trehalose-6-phosphate (T-6-P) formed was determined by anthrone color reagent after neutralizing all other sugars. Final solution was spectrophotometrically analyzed at A_{625} nm(Chaudhuri et al., 2008). Trehalose (13.2mM) was used as substrate for AT and NT activities. Incubations were for 15 min at 30 °C. Glucose liberated enzymatically was determined by glucose oxidase- peroxidase (GOD-POD) method (Bergmeyer H.U, 1974).

Unit of enzyme activity (U) was expressed in micromole (µmole) of Phosphate liberated (for TPP); µmole of T-6-P synthesized (for TPS) and µmole of reducing sugar liberated (for AT and NT), per min under assay conditions.

Protein estimations

The protein content of enzyme solutions was measured by Bradford Reagent as per technical bulletin provided by the manufacturer, Sigma, USA. Protein content of whole cell homogenate was determined by the modified method of Lowry (Bergmeyer and Bernt, 1974). Standard protein solution used was bovine serum albumin (BSA) from Sigma, USA.

Intracellular metabolite extraction and their estimation

Intracellular metabolites from known mass of cells were extracted with 0.5 N cold perchloric acid according to the protocol of Sutherland and Wilkinson, 1971. AdoMet was estimated by HPLC using a cation-exchange column Partisil 10 SCX (4.6 × 250 mm, Whatman, England) (Biswas and Ghosh, 1997). Known volume of metabolite extract was injected, and amount of AdoMet present was determined from standard curve generated by injecting standard AdoMet solutions. AdoHcy was also determined from the same chromatograms by injecting AdoHcy standards, and comparing peak areas of AdoHcy of injected samples with standard curve. Trehalose was measured following the protocol of Parrou and Francois, 1997 using purified AT by DEAE Sephadex A50 column chromatography. Intracellular trehalose was also quantified enzymatically by measuring amount of glucose produced due to hydrolysis of trehalose by purified acid trehalase (Chaudhuri et al., 2008). Intracellular metabolite content was measured as µmole (g wet wt)$^{-1}$.

Study of Trehalose Metabolism during different stages of Growth

Yeast cells were grown in YPD medium, aliquots were collected at different hours of growth to get A_{660} ~10, A_{660} ~15, A_{660} ~20, A_{660} ~25, A_{660} ~30, and A_{660} ~35. The cells of different absorbance values were then separately harvested by centrifugation. In each case, total cell mass was divided into two parts. One part was kept for enzyme preparation and another part was used for metabolite extraction by perchloric acid. The enzymes TPS, TPP, AT, NT and the intracellular metabolites trehalose, glucose, glucose 6 phosphate, AdoMet and AdoHcy were extracted and measured.

Biosynthesis of Trehalose during incubation of yeast cells with 1 mM AdOx and AdoMet [short term incubation]

YPD grown cells (A_{660}~20) were harvested by centrifugation and re-suspended under shaking at 30°C in 25mM MES-KOH buffer,[pH6.0] containing either 1 mM AdOx or AdoMet. The cells were incubated for 1 hour in presence of the chemicals. Aliquots were collected at different intervals in ice cold water to terminate the reactions and centrifuged at 5000 x g for 5 min to collect the cell mass. Cells were washed thoroughly to remove all traces of adhering chemicals and stored at 0-4°C. A portion was used for crude enzyme preparation while another portion was used for intracellular metabolite extraction. (Bhattacharyya et al, 2005)

Biosynthesis of Trehalose during incubation of yeast cells with 0.1 mM Adox and AdoMet [long term incubation]

YPD grown cells from diauxic phase (A_{660} ~20) were harvested and the total cell mass was divided into three parts. Of the three parts, one part was re-suspended in fresh YPD medium in absence of Adox and AdoMet (control) and the other two parts were resuspended in medium containing either 0.1 mM AdOx or AdoMet. Those cells were harvested at different intervals of 4, 8, 24, 48 hr. Aliquots collected were washed and stored at 0 - 4 °C for further studies. Trehalose metabolic profile, including intracellular trehalose level and activities of trehalose metabolic enzymes was monitored. Intracellular AdoMet and AdoHcy content were also measured by HPLC method. (Bhattacharyya et al, 2005).

Results

Study of Trehalose Metabolism during different stages of Growth

The results indicate that the intracellular trehalose content was found maximum at O.D 20 and at that particular growth stage the activity of the trehalose synthesizing enzymes. TPP and TPS were also highest. The activity of the hydrolyzing enzyme AT was found to be very low nearly 350 times lower than TPS activity and NT was found to show the lowest activity. Concurrent with this, the intracellular Glucose level was low showing that rate of anabolism was much greater than the catabolic activity. [Fig: 1]

Biosynthesis of Trehalose during incubation of yeast cells with 1 mM AdOx and AdoMet [short term incubation]

Trehalose content increased during AdoMet treatment

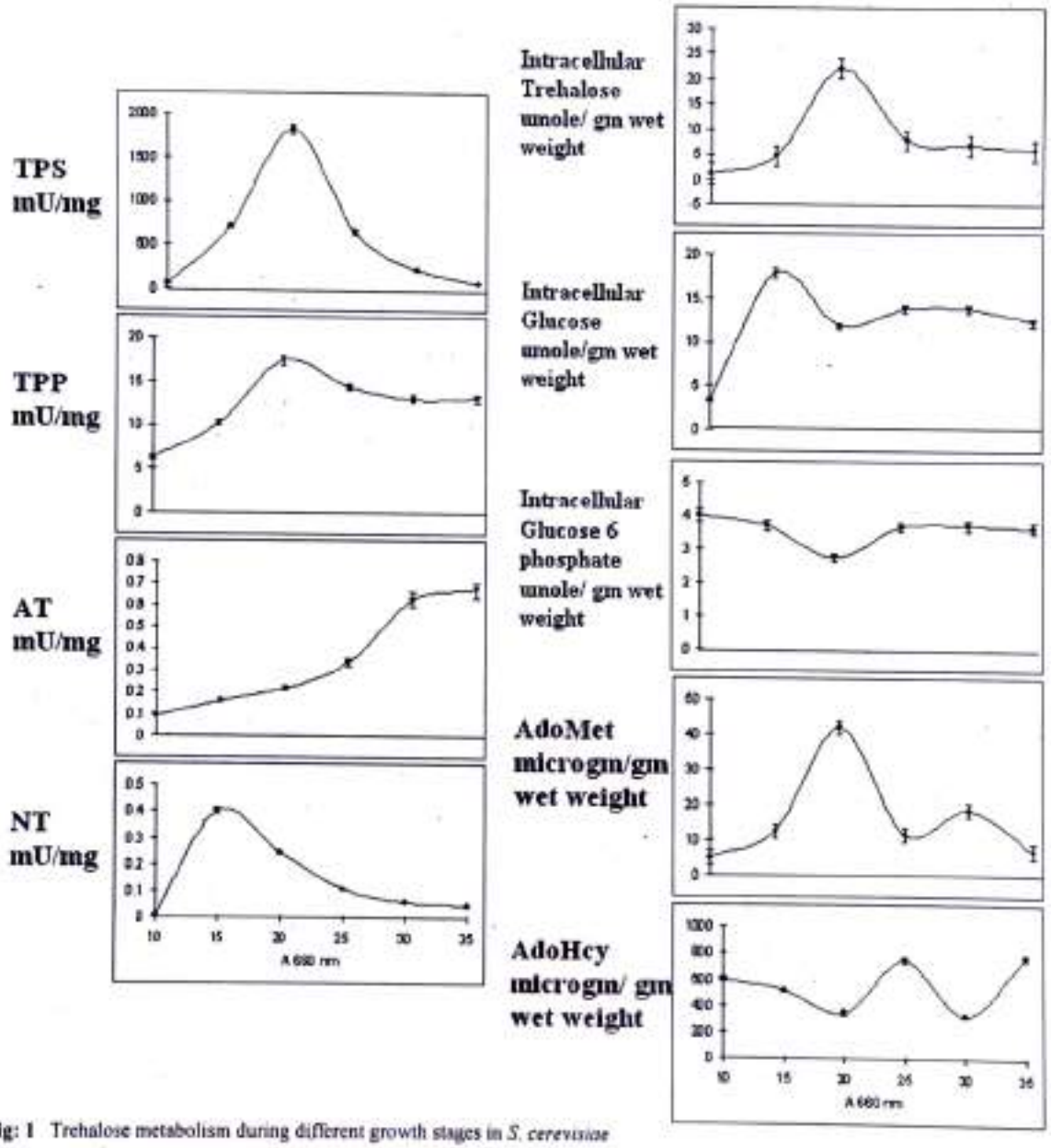

Fig: 1 Trehalose metabolism during different growth stages in *S. cerevisiae*

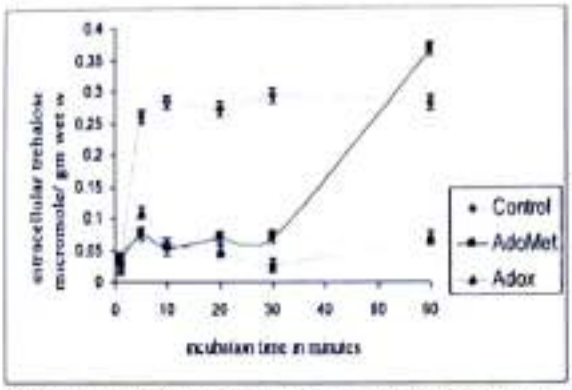

Fig: 2 Effect of short term incubation on trehalose content of yeast cells in 1 mM AdOx and AdoMet treatment. Yeast cells at A_{600} ~ 20 were suspended in MES-KOH buffer containing 1mM AdOx, AdoMet or water and incubated for 1 h. Level of trehalose of aliquots collected at 1, 5, 10, 20, 30 and 60 min of incubation was monitored. Data given are the mean of at least three sets of experiments.

from 20 mins onwards. [Fig: 2]

G6P content increased upto 10 mins and then decreased in AdoMet treated cells. Intracellular Glucose level was found to remain lower throughout the period of incubation in AdoMet treatment than both Adox treated and Control sets. Intracellular AdoMet and AdoHcy level was opposite to each other during the different treatments, while AdoMet content increased in AdoMet treated sets over Adox treatment and Control cells. [Table: 1]

TPS content increased during AdoMet treatment over Adox treated cells. But both were lower than Control sets. TPP activity peaked during AdoMet treated sets over Adox treated cells, but both showed lesser specific activity than control sets. [Table: 2]

Biosynthesis of Trehalose during incubation of yeast cells with 0.1 mM Adox and AdoMet [long term incubation]

Intracellular trehalose level of Adox treated cells were seen to be lower than Control throughout the period of incubation. Trehalose level initially decreased upto 4 hours in all the sets. Thereafter the cells with AdoMet treatment showed sharp increase in trehalose content, i.e., in 8 hours 3 fold, in 24 hours nearly 5 fold increases with respect to Adox treated and Control sets. But at the point of 48 hours, cells reached stationary phase and trehalose level increased irrespective of treatment. From 4 hour incubation onwards in both the synthesizing enzymes TPS and TPP, specific activity was found to increase in AdoMet treated cells over Adox treated sets and Control sets. AT and NT was found to show lower activity in AdoMet treatment over Adox treatment. Intracellular AdoMet content increased in AdoMet treated sets over Adox treatment and Control cells [Fig: 3] while intracellular AdoHcy level was found to be just opposite to AdoMet content. [Table 3]

Intracellular Glucose and Glucose 6 phosphate levels were higher in Adox treatment over AdoMet treatment throughout the period of incubation. [Table: 3]

Discussion

During the growth of yeast, trehalose accumulation begins at the diauxic shift, continues till entering stationary phase and degradation of trehalose starts once cells have entered stationary phase (Werner- Washburne et al. 1993). In *S. cerevisiae*, trehalose is synthesized by a two step pathway that requires trehalose synthase

Table 1: Effect of short term incubation on intracellular glucose, G-6-P, AdoMet and AdoHcy contents of yeast cells in 1 mM AdOx and AdoMet treatment. Yeast cells at A_{600} ~ 20 were suspended in MES-KOH buffer containing 1mM AdOx, AdoMet or water and incubated for 1 h. Level of trehalose of aliquots collected at 1, 5, 10, 20, 30 and 60 min of incubation was monitored. Data given are the mean of at least three sets of experiments. AX=Adox, AM=AdoMet, C=Control

Incubation Time in mins	Intracellular Glucose*			Intracellular G-6-P*			Intracellular AdoMet*			Intracellular AdoHcy*		
	AX	AM	C	AX	AM	C	AX	AM	C	AX	AM	C
1	0.0508	0.0479	0.0574	2.1534	2.1171	2.4034	415.22	368.97	257.81	649.32	442.21	558.67
5	0.0428	0.0366	0.099	2.1145	2.1562	2.3339	249.52	445.83	234.35	1033.04	201.32	944.88
10	0.044	0.0379	0.0576	2.1617	2.2145	2.07	144.18	627.52	154.29	743.92	543.71	933.12
20	0.0471	0.0371	0.0469	2.2062	2.0839	2.1284	189.13	1417.04	114.65	717.11	443.42	2351.17
30	0.0442	0.037	0.0527	2.2006	2.2228	2.2312	88.58	1467.31	59.85	645.84	433.5	349.59
60	0.047	0.0394	0.0489	2.2228	2.195	2.3034	48.15	319.21	67.04	1237.67	744.29	1214.53

*µmole per gram wet weight

Table 2: Effect of short term incubation on trehalose biosynthesizing enzymes TPP, TPS, AT and NT contents of yeast cells in 1 mM AdOx and AdoMet treatment. Yeast cells at A_{600} ~ 20 were suspended in MES-KOH buffer containing 1mM AdOx, AdoMet or water and incubated for 1 h. Level of trehalose of aliquots collected at 1, 5, 10, 20, 30 and 60 min of incubation was monitored. Data given are the mean of at least three sets of experiments. Ax=Adox, AM=AdoMet, C=Control

Incn.§	TPP*			TPS*			AT#			NT#		
	AX	AM	C	AX	AM	C	AX	AM	C	AX	AM	C
1	1.09	20.06	26.02	762.87	165.83	203.18	8.62	1.46	5.62	6.11	1.007	18.11
5	1.19	17.78	17.65	934.58	439.18	3812.41	1.54	0.97	9.49	2.94	1.07	14.41
10	15.74	17.08	41.38	954.3	1460.44	1943.76	3.75	0.86	4.4	3.35	1.66	4.66
20	18.94	22.99	25.66	264.4	727.21	2590.85	3.63	0.73	10.74	3.42	1.9	3.66
30	15.84	25.93	27.33	118.55	244.67	618.43	2.86	0.77	6.63	2.58	1.66	3.78
60	21.98	17.17	17.88	304.71	1508.35	11109.29	3.34	0.9	7.4	5.97	1.36	18.62

*U/mg, # mU/mg ; §Incubation time in minute

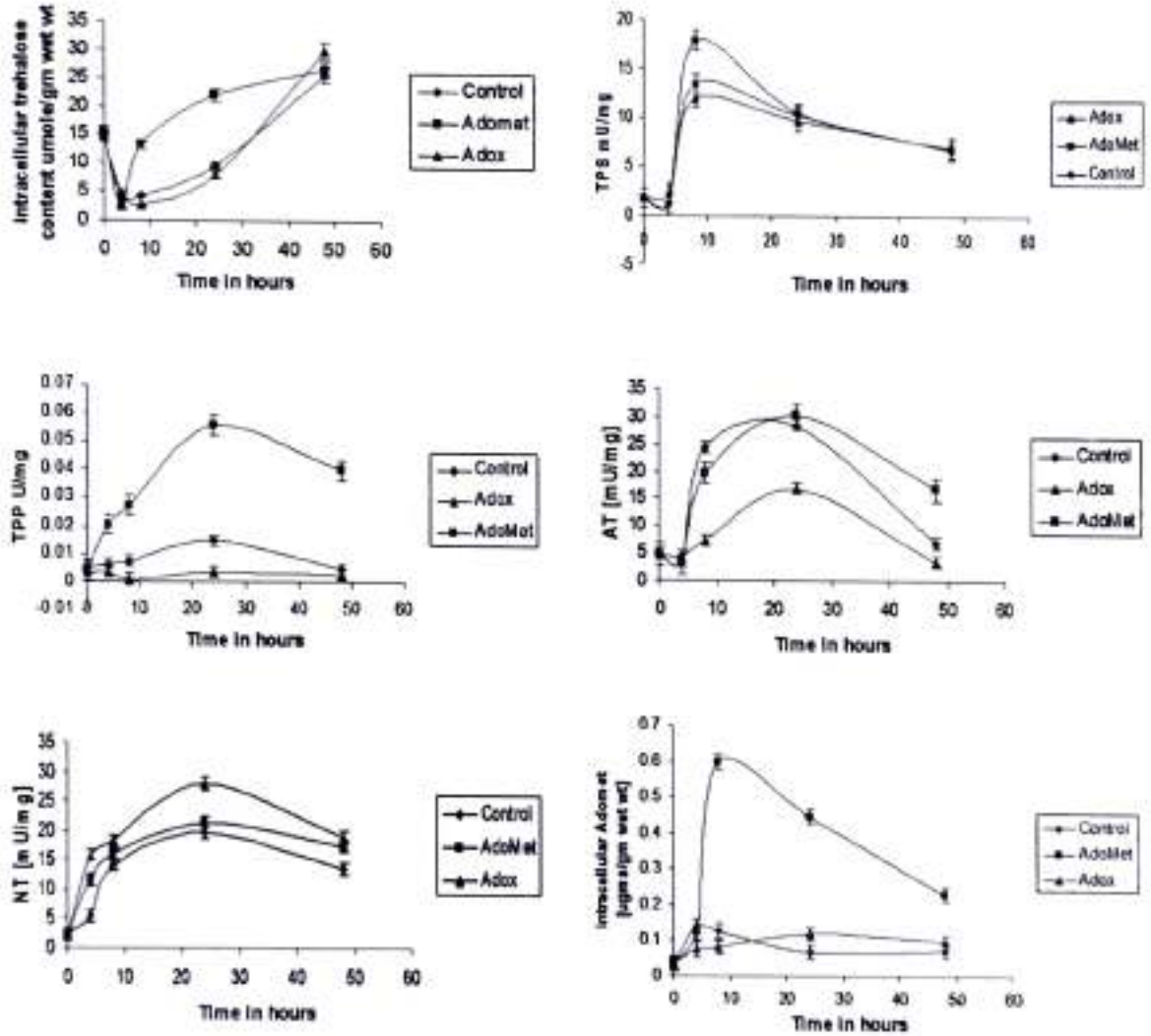

Fig 3: Effect of AdoMet and Adox on Trehalose metabolism during 48 hours incubation. YPD grown cells from A_{600} ~20 were harvested by centrifugation and re-suspended under shaking in fresh YPD medium containing 0.1 mM AdOx or AdoMet for 48 h incubation. In either case a positive control (minus added chemicals) was maintained. After different time intervals, aliquots were collected in ice-cold water to terminate the reactions and centrifuged in cold at 10,000 × g to obtain cell pellets. Changes in stored trehalose level and corresponding enzyme activities of TPS, TPP, AT and NT, and intracellular AdoMet level of aliquots collected at 4, 8, 24 and 48 h were studied. Data given are the mean of at least three sets of experiments.

enzyme complex which consists of (i) trehalose-6-phosphate synthase (TPS) subunit AND (ii) trehalose-6-phosphate phosphatase (TPP) subunit. Trehalose-6-phosphate is formed from Glucose-6-phosphate (G6P)

Table 3: Effect of AdoMet and Adox on Trehalose metabolism during 48 hours incubation. YPD grown cells from A_{660} ~20 were harvested by centrifugation and re-suspended under shaking in fresh YPD medium containing 0.1 mM AdOx or AdoMet for 48 h incubation. In either case a positive control (minus added chemicals) was maintained. After different time intervals, aliquots were collected in ice-cold water to terminate the reactions and centrifuged in cold at 10,000 × g to obtain cell pellets. Changes in intracellular glucose, G-6-P and AdoHcy levels of aliquots collected at 4, 8, 24 and 48 h were studied. Data given are the mean of at least three sets of experiments. Ax=Adox, AM= doMet, C=Control

Incubation time (h)	Intracellular Glucose*			Intracellular G-6-P*			Intracellular AdoHcy#		
	AX	AM	C	AX	AM	C	AX	AM	C
0	0.00134	0.00166	0.00199	3.002	3.258	3.3	0.1452	0.16729	0.1672
4	0.00134	0.00134	0.00067	3.9856	3.574	3.15	0.40081	0.19017	0.38566
8	0.00199	0.0025	0.000335	3.952	3.692	3.119	0.1862	0.38555	0.256
24	0.00299	0.0025	0.0032	4.675	3.149	3.169	0.1318	0.2263	0.2052
48	0.00366	0.00299	0.0035	3.0653	3.01	3.778	0.1388	0.0862	0.26965

*μmole/gm wet weight, #μgm/gm wet weight

and Uridine 5' diphosphoglucose (UDPG) by TPS which is then dephosphorylated to trehalose by TPP. The enzyme responsible for trehalose hydrolysis is trehalase existing in two different forms; one is cytosolic neutral trehalase (NT) and the other one is vacuolar acid trehalase (AT) (Cabib and Leloir, 1958). Since the synthesis of trehalose is catalyzed by the enzyme TPS and TPP, level of trehalose in the cells is dependent on the activity profile of the enzymes. Observation from our present study indicates an increase in TPS and TPP activity from exponential phase of growth (A_{660} ~ 10) till early diauxic phase (A_{660} ~20), and decrease in TPS and TPP activity with entry into stationary phase, corresponding to the increase in intracellular trehalose level throughout the growth of the yeast cells.

The intracellular trehalose level increased with time in the yeast cells incubated with AdoMet and decreased when treated with Adox with respect to control throughout the period of incubation. Initially, the trehalose level decreased upto 4 hours in both the treatments (AdoMet and Adox) and this may be due to the fact of complete utilization of pre-synthesized trehalose already present in the cell. Thereafter, the cells incubated with AdoMet showed sharp increase in trehalose content. As the cells had reached the stationary phase at 48 hours, trehalose level showed an increase irrespective of the treatment.

Concurrent with this observation the two trehalose synthesizing enzymes, TPS and TPP activity were higher during AdoMet treatment with respect to Adox indicating the possible effect of Methylation on trehalose biosynthesis. In case of the hydrolyzing enzymes, AT and NT, the effect of AdoMet was found lower than Adox which suggests no prominent effect of methylation on the activity of the hydrolyzing enzymes. Intracellular Glucose level was found very low due to this reduced hydrolysis of trehalose. This could be attributed to the fact of glucose getting converted to G6P in the first step of Glycolysis. Moreover, the variation of TPS and TPP activity and intracellular trehalose content in different treatments has been supported by the intracellular AdoMet and AdoHcy contents which have been determined by HPLC. This may be justified with the mechanism of a methylation reaction of proteins in which a protein is transformed into their methyl esters where methyl group donor is AdoMet. During the reaction AdoMet gets converted to AdoHcy (Adenosine Homocysteine), which by further hydrolysis splits up into Adenosine and Homocysteine residues by the enzyme AdoHcy Hydrolase. Adox plays a role as an indirect trans-methylation inhibitor. It blocks the conversion of AdoHcy to its components and thus in cells treated with Adox, the level of AdoHcy increases. This AdoHcy is a competitive inhibitor of all Methyl Transferases. Thus the transfer of Methyl groups from AdoMet to Protein stops. Adox thus causes indirect inhibition of methylation. The gene encoding PIMT is missing in *S. cerevisiae*, but our findings clearly indicate an elevation in trehalose biosynthesis by methylation. This leads us to conclude that there is possibility of presence of a new enzyme responsible for methylation of either TPS or TPP or both in *S. cerevisiae*.

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