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Possible role of histidine in the L-proline transport system of *Saccharomyces cerevisiae*

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The L-proline transport system of *Saccharomyces cerevisiae* is shown to be specifically inactivated upon incubation of intact yeast cells with the histidine modifier diethylpyrocarbonate. The extent of inactivation is half-maximum at 0.5 mM diethylpyrocarbonate for an incubation of 2 min at 30°C and pH 6.0. Under the same conditions, the time dependence of inactivation is monophasic with the second-order rate constant of 5.5 $M^{-1} \cdot s^{-1}$ and the maximum rate J_{max} of L-proline transport is lowered by about 50%, while the K_T value remains unchanged. Moreover, L-proline afforded significant protection against diethylpyrocarbonate inactivation. The complete reactivation of a partially inactivated L-proline transport system by neutral hydroxylamine and the elimination of the possibility that the modification of other amino acid residues are responsible for the inactivation, suggested that the transport protein inactivation occurs solely by a modification of histidine residues.

Introduction

L-Proline and possibly L-alanine are the only natural substrates of the high-affinity active transport system in *Saccharomyces cerevisiae* [1–3]. A sharp pH optimum of L-proline transport at pH 5.8–5.9 [2] suggests that the 'active site' of the corresponding transport protein(s) may require a certain degree of protonation to be fully functional. Moreover, a plot of log J_{max}/K_T vs. pH yields two pK_a values at pH 6.8 and 4.9 [4].

To understand the L-proline transport mechanism, we investigated the chemical nature of amino acids ionizable at such pH values by a detailed in vivo study of the reactions of the L-proline transport system with different group-specific modifiers before measuring the initial rates of L-proline transport. The inactivation of the system by diethylpyrocarbonate suggesting that histidine residues of the appropriate transport protein may be involved in the specific binding and/or translocation of L-proline into the yeast cells is reported here.

Materials and Methods

Microorganism. Culture conditions of facultatively anaerobic aneuploid strain *Saccharomyces cerevisiae* K (CCY 21-4-60) were as previously described [3].

Initial rate of L-proline transport. After a 1-h incubation with 1% glucose which stimulates the synthesis of the L-proline transport system [5], the yeast cells (5–7 mg dry wt. per ml) were washed with distilled water and resuspended in 50 mM phosphate buffer at pH 6 (buffer A). After 2 min

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preincubation with 0.4 mM cycloheximide at 30°C in a Dubnoff incubator, 0.05 mM ^{14}C-labelled L-proline was added and 0.2 ml samples of the cell suspension were withdrawn at intervals and filtrated through Synpor 5 (0.6 μm pore diameter) filters. The cell pellet on the filter was twice washed with 1 ml of ice-cold buffer A and transferred to a scintillation vial with a toluene-plus-ethanol scintillation cocktail.

Modification with diethylpyrocarbonate. The yeast cells (5–7 mg dry wt. per ml), preincubated with 1% glucose for 1 h, were resuspended at 30°C in buffer A and diethylpyrocarbonate was added as an ice-cold ethanolic solution (final ethanol concentration was 1% v/v). The reaction was allowed to proceed with agitation for 2 min and quenched by a 50-fold dilution with buffer A. The final diethylpyrocarbonate concentration was too low to interfere with the subsequent transport assay.

In reactivation (e.g. decarbethoxylation) experiments the yeast cells with diethylpyrocarbonate-inactivated L-proline transport system were resuspended in 50 mM phosphate buffer (pH 7.3) plus hydroxylamine and incubated for up to 15 min. The reaction was stopped by a 50-fold dilution with buffer A and L-proline transport activity was determined. Control experiments showed that about 25% of the L-proline transport activity was lost in 15 min on exposure to hydroxylamine. Therefore, this value was normalized to represent 100% activity.

Treatment with other group-specific modifiers. Glucose pretreated cells were incubated for 15 min at 30°C in an appropriate buffer to which the respective group-specific modifier was added at different concentrations immediately before use (for details see Table I).

Chemicals. Cycloheximide was obtained from Fluka (Switzerland), DCCD was purchased from British Drughouses (U.K.) and *N*-ethylmaleimide was the product of Koch-Light Genzyme (U.K.). The remaining group-specific modifiers were purchased from Sigma (U.S.A.). All other inorganic and organic chemicals were of analytical grade and were obtained from Lachema (Czechoslovakia). Uniformly ^{14}C-labelled L-proline was from the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia).

Results

While the 2,4,6-trinitrobenzenesulfonic acid, pyridoxal 5′-phosphate, *N*-acetylimidazole, phenylmethylsulfonyl fluoride and 2,3-butanedione failed to modify the L-proline transport activity, the remaining modifiers tested were effective at reasonably low concentrations (Table I). To distinguish between their direct and indirect interactions with the L-proline-specific transport system, we protected the system against the inactivation by modifiers through simultaneous addition of L-proline at saturation concentrations. As may be seen from Table I, only the inhibitory effect of diethylpyrocarbonate (ethoxyformic anhydride), a fairly specific modifier of the side chain of histidine at pH 6 [12,17], was significantly diminished by the presence of L-proline in the reaction mixture. This suggested that a more detailed investigation of the reaction of the diethylpyrocarbonate with L-proline transport system would be of value.

Incubation of intact yeast cells in phosphate buffer with diethylpyrocarbonate resulted in a rapid loss of L-proline transport activity. The percentage inactivation was only slightly pH-dependent at acidic and neutral pH and increased more steeply at alkaline pH values (Fig. 1). All further data were obtained after incubation with the modifier at pH 6.0, since this pH value afforded reasonable sensitivity to diethylpyrocarbonate and was rather close to the optimum for L-proline transport [2].

Some kinetic properties of the partially inactivated L-proline transport system were studied (Fig. 2). In the absence of modifier, the K_T and J_{max} values of L-proline transport are 29 μM and 40 nmol · s^{-1} · (g dry weight)$^{-1}$, respectively. After partial inactivation by 0.5 mM diethylpyrocarbonate and following elimination of unbound modifier, the K_T of L-proline transport was unchanged while the J_{max} was reduced to about 50%.

Moreover, the degree of inactivation was dependent on diethylpyrocarbonate concentration in such a way that half-maximum inactivation was produced by 0.5 mM diethylpyrocarbonate using incubation at 30°C of the yeast cells with the modifier (Fig. 3).

Fig. 4A shows that the reaction of the L-proline

TABLE I

EFFECTS OF GROUP-SPECIFIC MODIFIERS ON L-PROLINE TRANSPORT

I_{50}, modifier concentration causing 50% inactivation of the initial rate of transport under the conditions described in Materials and Methods. DCCD, *N,N'*-dicyclohexylcarbodiimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Mes, 4-morpholine-ethanesulfonate.

Main target group	Inhibitor	Buffer	I_{50} (nmol·l^{-1})	Protection by L-proline
α- and ε-amino	2,4,6-Trinitrobenzene sulfonic acid [6]	50 mM carbonate (pH 9.4)	n.i.	-
ε-Amino	Pyridoxal 5'-phosphate [7]	50 mM borate (pH 8.3)	n.i.	-
Carboxyl	DCCD [8]	10 mM Mes (pH 5.2)	0.1	no
	EDAC	50 mM Mes (pH 5.0)	0.35	no
Tyrosine hydroxyl	*N*-Acetylimidazole [9]	10 mM Mes (pH 7.1)	n.i.	-
Serine hydroxyl	Phenylmethylsulfonyl fluoride [10]	50 mM phosphate (pH 7.0)	n.i.	-
Imidazolium	Diethylpyrocarbonate [11–13]	50 mM phosphate (pH 6.0)	0.5	yes
Guanidinium	Phenylglyoxal [14]	25 mM diethylbarbiturate, (pH 6.9)	5.0	no
	2,3-Butanedione [15,16]	50 mM Mes + 50 mM borate, (pH 7.0)	n.i.	-
Sulfhydryl	*N*-Ethylmaleimide [17]	10 mM Mes (pH 7.1)	0.4	no

n.i., no inhibitory effect.

transport system with diethylpyrocarbonate exhibits a pseudo-first-order time-dependent loss of the L-proline transport activity at 30°C. The apparent first-order reaction constant varied as a function of the modifier concentration (Fig. 4B), indicating that the reaction obeys true second-order kinetics. The value of the second-order rate

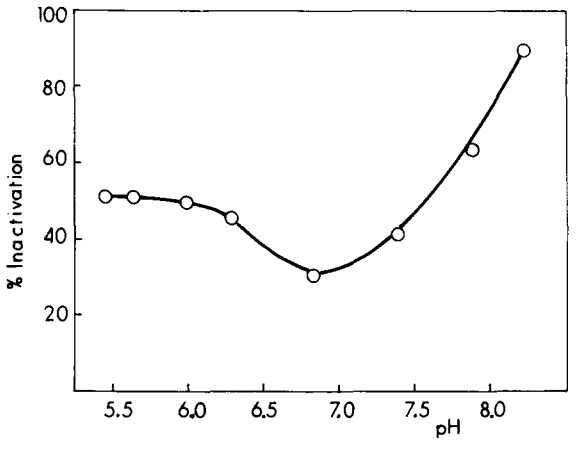

Fig. 1. pH dependence of the L-proline transport inactivation by diethylpyrocarbonate. Yeast cells (6.5 mg dry wt. per ml) were suspended in 50 mM phosphate buffer at the pH indicated and 0.5 mM diethylpyrocarbonate was added. The inactivation and transport assays were performed as described in Materials and Methods.

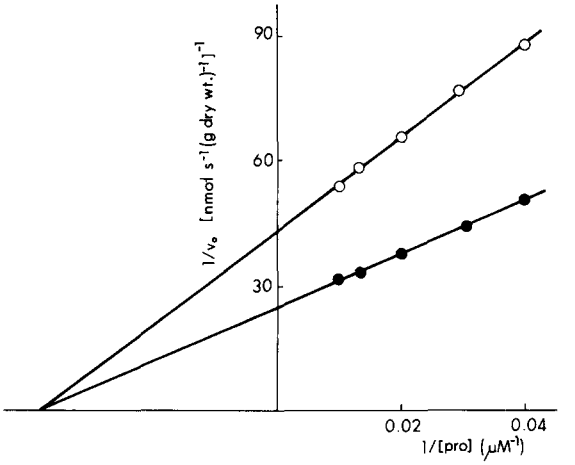

Fig. 2. Effects of modification by diethylpyrocarbonate on the kinetic parameters of L-proline transport. Yeast cells (6.8 mg dry wt. per ml) were first modified (○) or not (●) by incubation with 0.5 mM diethylpyrocarbonate and the activity of the modified and unmodified L-proline transport system was measured in the assay medium containing increasing concentrations of L-proline.

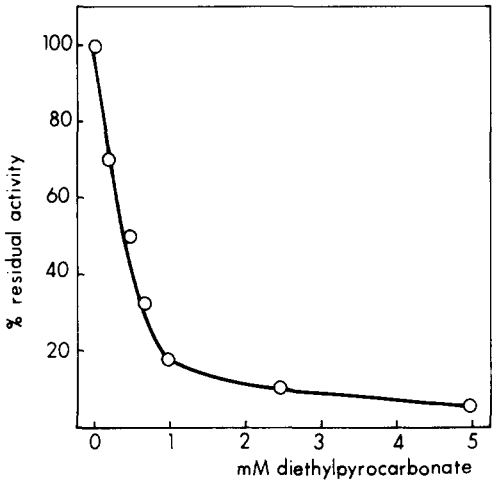

Fig. 3. Inactivation of L-proline transport as a function of diethylpyrocarbonate concentration. Yeast cells (6.3 mg dry wt. per ml) were incubated in the presence of various diethylpyrocarbonate concentrations and the residual transport activity was measured as described in the text.

constant derived from Fig. 4B was 5.5 $M^{-1} \cdot s^{-1}$. The slope of the double log plot of k_s (pseudo-first-order rate constant for inactivation) against diethylpyrocarbonate concentration gives the number of diethylpyrocarbonate molecules reacting per corresponding transport protein molecules in the inactivation of transport activity [18]. The slope of the line in Fig. 4B (inset) is 1.1 indicating that the modification of approximately one histidine residue is sufficient to abolish the ability of the transport protein to mediate L-proline transport.

The reversibility of the inactivation with hydroxylamine is shown in Fig. 5. The L-proline transport activity lost by diethylpyrocarbonate treatment was practically fully restored by subsequent 15 min incubation with 50 mM hydroxylamine.

Finally, Fig. 6 demonstrates that the incubation of intact yeast cells with L-proline at saturation

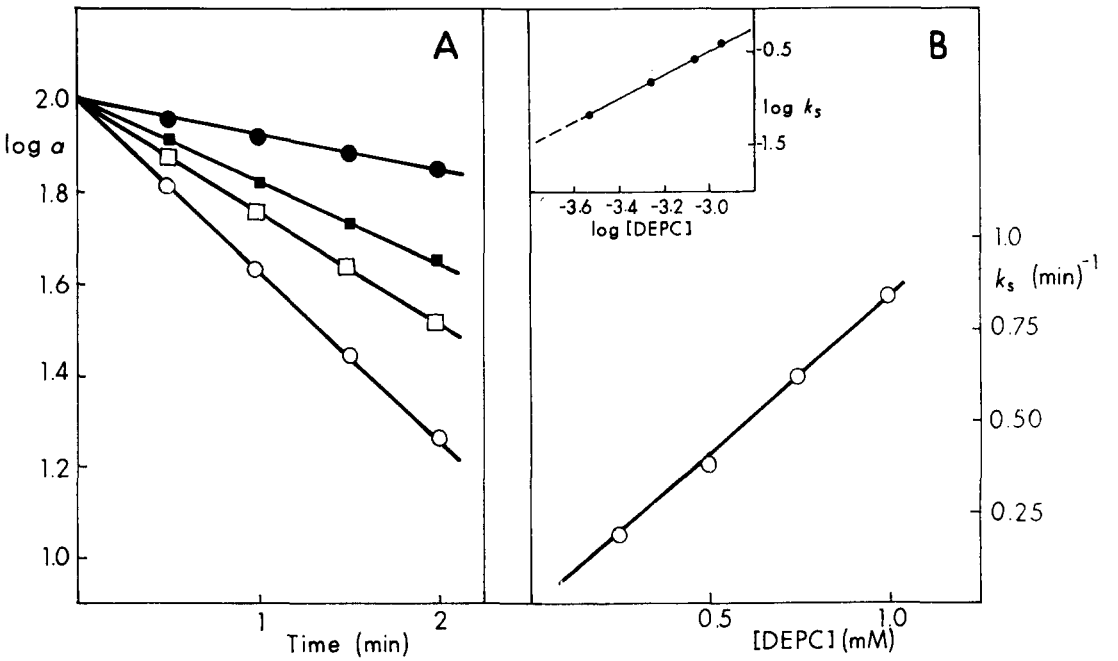

Fig. 4. (A) Kinetics of inactivation by diethylpyrocarbonate during time. Yeast cells (5.1 mg dry wt. per ml) were incubated with 0.25 mM (●), 0.50 mM (■), 0.75 mM (□) and 1.0 mM (○) diethylpyrocarbonate (DEPC) at pH 6.0 under the conditions described in the text. (B) Dependence of the pseudo-first-order rate constants for the inactivation on the concentration of diethylpyrocarbonate. Values of k_s were determined from the slopes of the semilogarithmic plot of residual activity a versus time in Fig. 4A. Inset: a plot of log k_s versus log [diethylpyrocarbonate].

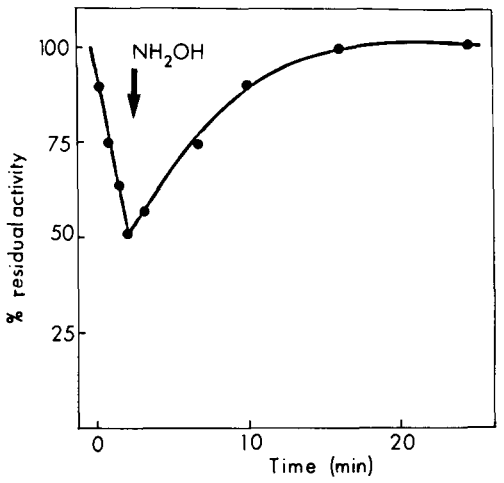

Fig. 5. Reactivation of partially inactivated L-proline transport system by neutral hydroxylamine. Yeast cells (6.1 mg dry wt. per ml) with the L-proline transport system possessing 50% of its original activity were washed and resuspended in 50 mM phosphate buffer (pH 7.3), 50 mM hydroxylamine was added and tested for reactivation at the times indicated on the abscissa.

concentrations (0.5–1 mM), either prior to the modification reaction by diethylpyrocarbonate or simultaneously with the modifier, abolish the inactivation significantly. On the other hand, no protection was produced by hydroxy-L-proline and by other amino acids (data not shown) which are not substrates of the L-proline specific transport system [2].

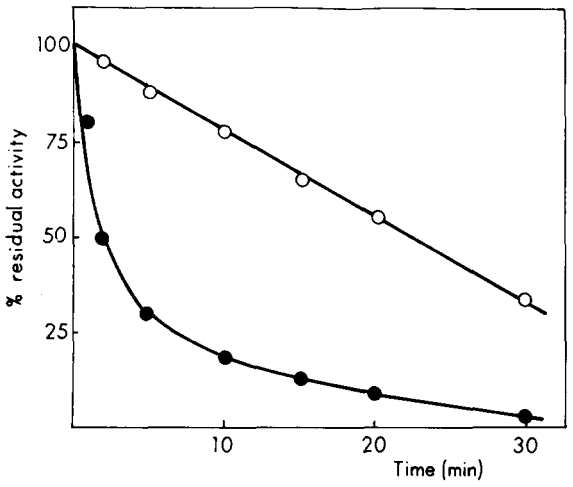

Fig. 6. Protection of the L-proline transport system by L-proline. Yeast cells (5.3 mg dry wt. per ml) were incubated with 0.5 mM diethylpyrocarbonate in 50 mM phosphate buffer (pH 6.0) alone (●) or with 1 mM L-proline (○) at the indicated time intervals. The residual transport activity was then measured as described in the text.

Discussion

Incubation of 'glucose-energized' yeast cells with diethylpyrocarbonate at pH 6.0 and 30°C results in a rapid loss of L-proline transport activity. The inactivation process conformed to pseudo-first-order kinetics and a single second-order rate constant of 5.5 $M^{-1} \cdot s^{-1}$ was estimated from the plot of log of residual activity versus time. The rate constant for the inactivation is only slightly higher than the values for the reaction of the imidazole ring in model compounds (2.3–3.5 $M^{-1} \cdot s^{-1}$) with diethylpyrocarbonate under the similar experimental conditions [19–21].

Second-order rate constants for the inactivation of various proteins by selective modification of their histidine residues with diethylpyrocarbonate at pH 6.0 have been found to range from about 0.18 $M^{-1} \cdot s^{-1}$ at pH 6.2, 26°C, for the inactivation of thermolysin [22], to 30 $M^{-1} \cdot s^{-1}$ at pH 6.0 and 20–25°C for the modification of hyperreactive histidine residues in pig-heart lactate dehydrogenase [19] and of yeast alcohol dehydrogenase [20]. Finally, Cousineau and Meighen [23] concluded that if the rate constants for the inactivation with diethylpyrocarbonate at pH 6 are sufficiently high (and here they appear to be), it may be reasonable to assume that the modification of a histidine residue is responsible. All these data taken together are thus consistent with a simple bimolecular reaction between diethylpyrocarbonate and a histidine residue of the L-proline transport system resulting in its inactivation.

Although the reaction of diethylpyrocarbonate with proteins is relatively specific at pH 6.0 for histidine residue, it can also modify arginine, lysine, cysteine, serine and tyrosine residues as well as α-amino groups of amino acids of various proteins under the same conditions [12]. While the modification of histidine and serine is reversible by hydroxylamine, the modification of tyrosine is more resistant to such reversal, and the modifications of lysine and cysteine residues are not reversible by hydroxylamine [12]. As shown in Table

I, the L-proline transport system is insensitive to the action of pyridoxal 5′-phosphate (10 mM), phenylmethylsulfonyl fluoride (1 mM), *N*-acetylimidazole (10 mM), 2,3-butanedione (50 mM) and 2,4,6-trinitrobenzenesulfonic acid (100 mM) and L-proline is unable to protect its own transport system against the *N*-ethylmaleimide and phenylglyoxal inactivation. Moreover, hydroxylamine efficiently and rapidly restores the activity of L-proline transport system inactivated with diethylpyrocarbonate (Fig. 5). It may be therefore concluded that with high probability the modification of the imidazole ring of a histidine residue, rather than of α- and/or ε-amino groups of lysine, hydroxyl groups of tyrosine and serine and of sulfhydryl groups of cysteine, is the cause of L-proline transport system inactivation by diethylpyrocarbonate.

The possibility of complete inactivation of the L-proline transport system by incubation with diethylpyrocarbonate indicates that chemically modified histidine residue is essential for the substrate-specific binding and/or translocation of L-proline across the plasma membrane. In addition, L-proline partially protects its own transport system against the diethylpyrocarbonate inactivation. If such protection is due to location of the reactive histidine in or near the 'active site' of the corresponding transport protein or to a change of conformation induced by binding to the active site of L-proline which would decrease the reactivity of essential histidine residue not located in the active site, remains to be ascertained.

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