Transport of dicarboxylates in *Saccharomyces cerevisiae*

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Transport of dicarboxylates plays an important role in cell metabolism. In particular, they are intermediates of the citrate cycle. Transport of succinate across the mitochondrial membrane provides correlation between metabolism in peroxysomes and in mitochondria. In recent years, much attention has been given to transport of dicarboxylates across plasma membranes of animal, plant and bacterial cells. However, yeast cell transporters have not been studied systematically. The results of our research group experiments for the period 2001-2010 years are summarized in this mini-review. Our previous experiments showed specific features of changes in levels of endogenous respiration substrates in *Saccharomyces cerevisiae* cells at low temperature. The rate of endogenous respiration of cells in the absence of exogenous substrates decreased exponentially with a half-period of about 5 h when measured at 30°C. This was associated with an indirectly shown decrease in the level of oxaloacetate in the mitochondria *in situ*. The rate of cell respiration in the presence of acetate and other exogenous substrates producing acetyl-CoA in mitochondria also decreased, whereas the respiration rate on succinate increased. These changes were accompanied by an at least threefold increase in the L-malate concentration in the cells within 24 h, indicating the physiological significance of L-malate in regulation of the *S. cerevisiae* cell respiration. A nonconventional approach to the measurement of succinate transport across plasmalemma is proposed. It is based on the conditions in which the succinate oxidation rate is limited by transport across plasmalemma. Transport of succinate into *S. cerevisiae* cells was determined using the endogenous coupled mitochondrial succinate oxidase system. The dependence of succinate oxidation rate on the substrate concentration was a curve with saturation. At neutral pH the K_m value of the mitochondrial "succinate oxidase" was fivefold less than that of the cellular "succinate oxidase". O-Palmitoyl-L-malate, not penetrating across the plasma membrane, completely inhibited cell respiration in the presence of succinate but not glucose or pyruvate. The linear inhibition in Dickson plots indicates that the rate of succinate oxidation is limited by its transport across plasmalemma. The plasma membrane of *S. cerevisiae* was found to have a carrier catalyzing the transport of dicarboxylates (succinate, L-malate and malonate). This approach allowed for the reproducible determination of K_m for the dicarboxylate transporter (7.3 ± 2.1 mM) within a half-hour period. We found that plasmalemmal dicarboxylate transporter is also involved in citrate influx and is modulated by pH and cations. Succinate and citrate transport into yeast cells was studied by measuring substrate oxidation rates in the presence and in the absence of effective impermeable oxidation inhibitors. Linearity of the Dickson plot for 2-undecyl malonate suggests that this inhibitor blocked the rate-limiting step upon oxidation of both substrates. This approach allowed fast (within 30–40 min) measurement of kinetic parameters of the transporter in individual samples. Succinate and citrate transport was insensitive to the protonophore FCCP, being activated by Na^+ ions and competitively inhibited by 2-undecyl malonate and K^+ ions. Values of K_i for 2-undecyl malonate were similar for both substrates. These data suggest that citrate and succinate influx is mediated by a common plasma membrane transporter. This is not typical for fungi. Topography of the active site of the *S. cerevisiae* plasmalemmal dicarboxylate transporter was studied using lipophilic derivatives of its substrates (2-alkylmalonates and O-acyl-L-malates). Probing of the active site of this transporter has revealed a large lipophilic area stretching between the 0.72 to 2.5 nm from the substrate-binding site. Itaconate inhibited the transport fivefold more effectively than L-malate. This suggests the existence of a hydrophobic region immediately near the dicarboxylate-binding site (to 0.72 nm). Fumarate but not maleate competitively inhibited succinate transport into the cells. It is suggested that the plasmalemmal transporter binds the substrate in the *trans*-conformation. The prospects of the proposed approach for scanning lipophilic profiles of channels of different transporters are discussed.

Keywords: endogenous cell respiration; transporters; L-malate; succinate; malonate; fumarate, itaconate, citrate, competitive inhibitors; O-acyl-L-malates; 2-alkylmalonates; *Saccharomyces cerevisiae*.

1. Introduction

Many C4-dicarboxylate transporters have been rather well studied. These transporters are found in various biological membranes. In recent years, much attention has been given to transport of dicarboxylates across plasma membranes of animal, plant and bacterial cells. However, yeast cell transporters have not been studied systematically. The known dicarboxylate transporters of the plasma membrane of yeasts, such as *Schizosaccharomyces pombe* [1], *Candida utilis* [2], *Kluyveromyces marxianus* [3], and *Pachysolen tannophilus* [4], transfer the substrate in symport with protons. The transporters, as a rule, in addition to L-malate and succinate, can also transport other dicarboxylates. Thus, the *K. marxianus* transporter can transfer D-malate, fumarate, and oxaloacetate [3] and the *S. pombe* transporter can transfer oxaloacetate, malonate, and maleate [5]. Sodium-dependent symporters of dicarboxylates have been studied in the plasmalemma of higher eukaryotes [6-8] but are unknown for yeasts. In *S. cerevisiae* plasma membrane a Na^+/H^+ antiporter and a Na^+-dependent symporter of phosphate ions are present [9, 10]. Therefore a Na^+ gradient can be created on this membrane under certain conditions (presence of NaCl in the medium or alkaline pH values).

The dicarboxylate transporters are present in the inner mitochondrial membrane [11], in the membrane of bacteria (in particular, *Serovar typhimurium* [12], *Treponema pallidum* [13], *Bradyrhizobium japonicum* [14]), and in the plasma membranes of higher (e.g. rabbit kidney [8]) and lower eukaryotes (*S. pombe* [5], *Kluyveromyces lactis* [15]). Molecules of mitochondrial transporters are twofold smaller than molecules of plasma membrane transporters [16]. Na^+/dicarboxylate symporters of higher eukaryotes contain ~600 amino acid residues [8, 16, 18], whereas H^+/dicarboxylate symporters of yeast contain ~440 residues [3, 5, 15] (*S. pombe* contains 438 amino acid residues [5]), which is nearer to the size of bacterial transporters (about 400 residues [12-14]). Mitochondrial transporters have six hydrophobic transmembrane α-helical segments [11, 17], but the plasma membrane transporters have 11 [8, 18] or 12 such segments [5, 15]. These hydrophobic areas are separated by hydrophilic regions exposed into solution [11, 19]. All six segments of adenylate transporter form a channel [17]. It is suggested that mitochondrial transporters of C_4-dicarboxylates may have the same structure [20]. The α-helical transmembrane regions of the plasma membrane transporter molecules encircle the channel by nearly two layers [21]. It is shown that four of 12 segments determine the K_m value for succinate of the rabbit liver Na^+/dicarboxylate symporter [18]. Only four of 12 segments form the inner surface of the human glucose transporter channel [21]. Hydrophobic segments of nearly all known C_4-dicarboxylate transporters have unit polar amino acid residues, but it is unclear whether they are exposed into the channel. The channels of some transporters of hydrophilic substrates are shown to have both a hydrophobic and hydrophilic inner surface (similarly to the glucose transporter [21] and the potassium channel of bacteria [22]). Three-dimensional structures of dicarboxylate transporters are still unstudied. For such transporters the probing of the channel near the substrate-binding site using inhibitors (amphiphilic derivatives of these hydrophilic substrates) should be very informative [23].

The work of our research group [23-29] was designed to study specific features of changes in the pool of endogenous substrates involved in the energy metabolism of *S. cerevisiae* under aerobic conditions at 0°C and the role of dicarboxylates in this process; to investigate transport of dicarboxylates across plasmalemma; to elucidate the role of a specific inhibitors, external pH, and concentration of cations on kinetic characteristics of this transport; to study substrate specificity and mechanism for the dicarboxylate transporter; to use O-acyl-L-malates and 2-alkyl-malonates for probing of its active site.

2. Specific features of changes in the pool of endogenous respiration substrates involved in the energy metabolism of *S. cerevisiae* [24]

The metabolism of reserve carbohydrates and respiratory function of mitochondria are interrelated [30-34]. The reserve carbohydrates, such as the polysaccharide glycogen and the disaccharide trehalose, are main sources of endogenous substrates for mitochondria. Biosynthesis of these reserve carbohydrates is activated by a decrease in the level of endogenous substrates [33, 35]. Their utilization increases upon exhaustion of endogenous metabolites, such as glucose-6-phosphate [36]. Interactions between the accumulation [36, 37] and utilization in the cell of carbohydrates under different stress conditions is of great interest [38-40]. Under aerobic conditions glycogen phosphorylase is activated immediately in the cells upon exhaustion of exogenous glucose [41]. The generated glucose-1-phosphate is converted during glycolysis to phosphoenolpyruvate (Fig.1). Pyruvate produced during the pyruvate kinase-catalyzed reaction is a key metabolite providing for the endogenous respiration of cells. Some amount of pyruvate is oxidized in mitochondria with production of acetyl-CoA, which is one of two substrates of citrate synthetase. Another fraction of pyruvate is converted to oxaloacetate (the other substrate of citrate synthetase) by pyruvate carboxylase. Thus, pyruvate produced from glycogen can assure the functioning of the citrate cycle in mitochondria and, respectively, the endogenous respiration of the cells. Cytosolic location of pyruvate carboxylase in *S. cerevisiae* [42] imparts a specific function to the malate dehydrogenase system (mitochondrial and cytosolic malate dehydrogenases), which is considered a transport system of reducing equivalents [43]. The main function of this system may be transport of oxaloacetate into the mitochondria. But this pathway is unable to support the anapleurotic purpose of oxaloacetate, because the equimolar replacement of oxaloacetate by malate occurring in this case [44] prevents this. However, the transport of L-malate by a dicarboxylate transporter [45] via exchange for phosphate (by an orthophosphate transporter [46]) can provide for the transport of substances compensating the diminution of mitochondrial metabolites during functioning of the citrate cycle.

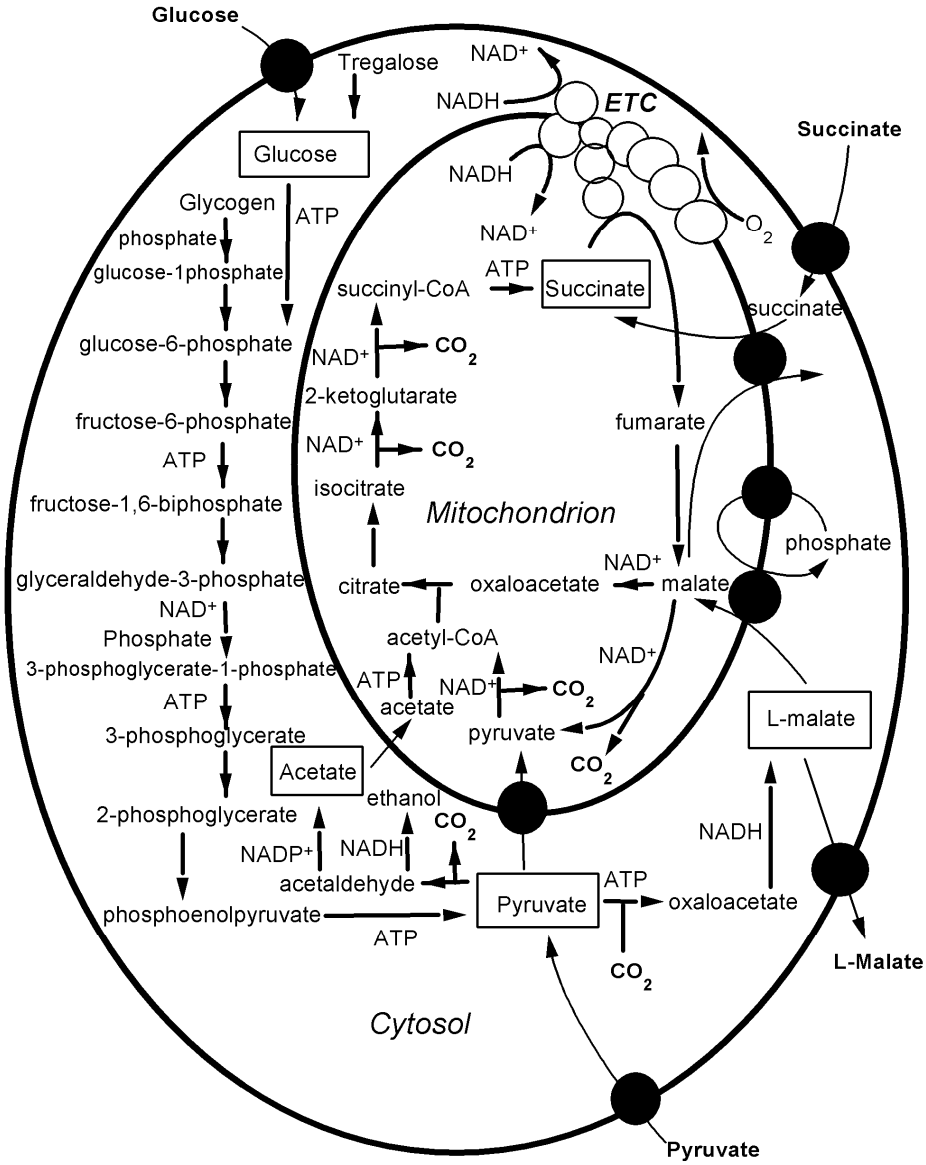

Fig. 1 Basic metabolism and the electron transport chain (ETC) in *S. cerevisiae* cells (scheme) [24].

S. cerevisiae cells were grown [24] under conditions favorable for proliferation of mitochondria: at low content of glucose [47]. The exponential growth phase terminated virtually concurrently with exhaustion of exogenous glucose (12-h culture). The endogenous respiration rate was high during this stage. In our experiments the initial rate of respiration which at 30°C was, on average, 16 nmol/min per mg dry weight of the cells [24] was significantly lower than the respiration rate in the presence of a saturating concentration of glucose (30-60 nmol/min per mg). Addition of pyruvate stimulated cell respiration three-fivefold. But exogenous L-malate poorly stimulated cell respiration: only for 19-45%. As it has been discussed, oxaloacetate required for functioning of the citrate cycle is produced in mitochondria from L-malate brought into them by the dicarboxylate transporter in exchange for phosphate. The oxaloacetate concentration in *S. cerevisiae* cells is very low (6-20 μM) [48]. We have determined [24] the level of mitochondrial oxaloacetate using an indirect approach based on the competitive interaction between oxaloacetate and malonate in the active site of succinate dehydrogenase [49, 50]. The level of endogenous oxaloacetate in the mitochondria decreased during the incubation of cells at 0°C. Thus, addition of malonate to freshly separated cells activated 1.3-1.7-fold their endogenous respiration due to displacement by malonate of oxaloacetate from the complex with succinate dehydrogenase [50, 51]. The effect of malonate weakened afterwards, and in 5-10 h of incubation of cells at 0°C malonate ceased to activate the endogenous respiration of the cells [24]. At the exogenous succinate concentration of 20 mM, 0.43 ± 0.03 mM malonate inhibited the cell respiration by 50% [24]. The decrease in the rate of endogenous respiration of the cells might be associated with a decrease in the L-malate level caused by the decrease in the content of glycogen. However, the decrease in the rate of endogenous respiration during the aerobic incubation at 0°C was accompanied by a significant increase in the level of L-malate (~threefold for 24 h) [24]. We showed that the concentration of L-malate increased not only in the cells, but also in the culture medium during the cells growth. Thus,

the L-malate concentration in the culture medium was increased by 21.6 ± 7.6 μM after 12 h of the cell growth. After 1.4 h the cell isolation, succinate activated the respiration only by 13%. However, with the increase in the L-malate level in the cells and decrease in the oxaloacetate concentration in the mitochondria *in situ*, the stimulating effect of exogenous succinate significantly increased. Thus, 13.6 h after the cell isolation, the rate of their respiration on the addition of succinate was increased 3.7-fold [24].

Under aerobic conditions the pool of endogenous substrates of *S. cerevisiae* cells was shown to change significantly during incubation at 0°C [24]. The exponential decrease in the respiration rate of the cells measured at 30°C after their incubation at 0°C suggests that at low temperature a process occurs with a rate fitting first order kinetics. Glycogen is the most likely substrate the level of which determines the rate of the process. During the aerobic incubation of the cells at 30°C [30] the level of glycogen is known to exponentially decrease. The phosphorolysis of glycogen may also occur under "softer" conditions, i.e., at 0°C. The decrease in the level of reserve carbohydrates would be correlated with the decrease in the levels of pyruvate and L-malate due to utilization of these substrates in mitochondria (Fig.1). The decrease in the rate of cell respiration along with the concurrent increase in the malate level in the cells might be explained by the increased level of malate in mitochondria leading to the increase in the level of oxaloacetate and, as a consequence, inhibition of succinate dehydrogenase and cell respiration. To explain our results [24], we supposed that at low temperature the anapleurotic function of malate (oxaloacetate) provided for by the malate transport via the mitochondrial dicarboxylate transporter should be disturbed (Fig.1). This disturbance seems to be caused by the significantly higher activation energy of the transporter-catalyzed transport than the enzyme-catalyzed reactions. Therefore, the rate of metabolite transport begins to determine the rate of their utilization on the temperature decrease from 30 to 0°C. This results in the decrease in the oxaloacetate and malate levels in the mitochondria and the increase in their levels in the cytosol. On the temperature increase from 0 to 30°C the acceleration of oxidation of the exogenous succinate with increase in the malate level in the cell can be due to the ability of malate not only to leave the cells but also enter the mitochondria in exchange for phosphate (Fig.1). The Chappell cycle is activated by the increase in the malate concentration in the mitochondria and the level of succinate increases due to exchange for this dicarboxylate. This is promoted by the significantly higher affinity of the mitochondrial dicarboxylate transporter for L-malate than phosphate [52]. Malate is produced in the cytosol due to pyruvate carboxylation. The mitochondria are the main source of carbon dioxide (Fig.1) and the L-malate concentration in the cell can be increased at the cost of the mitochondrial pool of the substrates. The mitochondrial transport of malate with increase in the temperature up to 30°C is not sufficient to recover this pool. We observed the decrease in the rate of oxidation of exogenous acetate and also glucose and pyruvate in such conditions [24]. These substrates could provide for the utilization of oxaloacetate needed for the oxidation of malate. But in order to activate this pathway, an efficient generation of the transmembrane potential in mitochondria is necessary. It should be mentioned that the transport of pyruvate catalyzed by the monocarboxylate transporter (accompanying cell respiration on glucose or pyruvate) is coimported with a proton [53, 54]. Acetate penetrates into the mitochondria by diffusion as acetic acid, i.e., its transport is also associated with the entrance of protons into the mitochondrial matrix. Thus, for oxidation of glucose, pyruvate, and acetate protons must be removed from the mitochondria. The removal of protons and ATP synthesis in mitochondria occur due to generation of the transmembrane potential. Acetate seems to promote the partial recovery of this pool in the early stages of exhaustion of the pool of mitochondrial substrates. Our findings [24] can be explained by the known properties of the enzymes and transporters functioning in *S. cerevisiae* cells. But the proposed interpretation is not the only possible one, and it needs be tested experimentally. This concerns, in particular, the role of the decarboxylating malate dehydrogenase located in the mitochondria of *S. cerevisiae* [55], which probably recovers the mitochondrial pool of the substrates. But the unique kinetic parameters of this enzyme excludes such a possibility. Its K_m for malate is ~50 mM. The role of the NAD redox-state in the control of the malate/oxaloacetate ratio in the cell compartments shoul be also discussed. The presence in the mitochondria of two NADH dehydrogenases, external and internal (Fig.1) maintains the balance between the NAD^+/NADH ratio in the cytosol and mitochondrial matrix. However, this process is not sufficiently understood at present. At pH 7 of the cytosol malate is a polar dianion with low permeability across the plasma membrane. The hypothesis about the removal of excess malate via simple diffusion of this dicarboxylate across the plasma membrane seems unlikely. Therefore, the metabolism is likely to be corrected by a dicarboxylate transporter of the plasma membrane similar to the transporter found in the yeast *K. lactis* [15]. The plasma membrane of *S. cerevisiae* is believed to lack a system of dicarboxylate transport [15, 56, 57]. The weak stimulation by malate and succinate of the freshly isolated cells in our experiments seems to favor this viewpoint. But the results obtained in the further stages of cell incubation at 0°C, when succinate considerably stimulates the malonate-sensitive respiration, contradicts this idea [24]. This suggests that the plasma membrane of *S. cerevisiae* has to contain a dicarboxylate transporter, the physiological function of which could be removal of excess malate from the cell. This viewpoint correlates with the data on L-malate secretion during the cell growth. In this connection, studies on the mechanism of dicarboxylate transport across the plasma membrane of *S. cerevisiae* seem to be especially interesting. We have shown [24] that after a incubation of *S. cerevisiae* cells at 0°C and pH 5.5 succinate significantly stimulates malonate-sensitive respiration (malonate inhibits mitochondrial succinate dehydrogenase).

3. Parameters of succinate transport in *S. cerevisiae* cells

A nonconventional approach to the measurement of succinate transport across plasmalemma is proposed [28]. It is based on the conditions in which the succinate oxidation rate is limited by transport across plasmalemma. As a tool to optimize conditions for the transport activity assay an impermeable specific inhibitor of plasma membrane dicarboxylate transporter was employed. Yeast culture was grown in synthetic medium under selected conditions [28]. After aerobic preincubation of *S. cerevisiae* cells at 0 ° C, the rate of endogenous respiration decreased substantially. It was stabilized during measurements at a level that was five times lower than oxidation rates in the presence of exogenous substrates. This approach allowed for the reproducible determination of K_m of the dicarboxylate transporter (7.3 ± 2.1 mM) within a half-hour period [28]. The proposed approach measuring the rate of transport of exogenous substrate inside the cell should satisfy the following demands: (1) The substrate oxidation rate should not vary in different experiments. (2) The rate of endogenous respiration should be low and stable during the measurements. (3) The rate of oxidation of the transported substrate should remain constant under transporter limiting conditions [28]. Kinetic parameters of transport measured with direct and indirect methods coincided under the above mentioned conditions.

The suggested method [28] is apparently efficient for the studying of low-activity transport systems in plasma membrane. Our indirect approach of plasmalemmal transport assay demonstrates a number of advantages compared to direct methods of metabolite transport measurement. The time of measurement at one substrate concentration was less than 5 min. The direct method of determination transport rate for each concentration point of the external substrate requires at least 1 h, and the method implicating radioactive substrate requires even longer [58]. At the same time, contrary to conventional direct methods this fast, but indirect, assay has some limitations. It can not be applied to the study of substances that permeate into the cell (like malonate and oxaloacetate [24]) and inhibit succinate oxidation in mitochondria. O-palmitoyl-L-malate permeates into the cell at pH 4.5, but at these pH values the limiting stage of succinate oxidation rate can not be determined with this inhibitor. The application for kinetic assay is limited when succinate is transferred in monoanionic form as for *S. pombe* [1]. For studying substrate transported into the cell, special conditions of cell cultivation and treatment should be tested. They should ensure minimization and stabilization of endogenous respiration, and the rate of exogenous substrate oxidation should be independent from the time of incubation at 0°C.

Contrary to direct methods of determination the affinity of dicarboxylate plasmalemma transporters from various yeast species [2-4], we defined the dependence for K_m calculation from a single curve plotted for 30 min. Linear dependence of succinate oxidation inhibition with O-palmitoyl-L-malate in Dickson coordinates indicated that the inhibitor targeted the limiting stage of substrate oxidation. Since the inhibitor did not permeate through the membrane during the experiment (20 min) [25], dicarboxylate transporter was the limiting factor. Thus, we monitored the rate of substrate transport across plasmalemma and its K_m measuring the rate of exogenous substrate oxidation in *S. cerevisiae* cells. The characteristic feature of our method of transport measurement across plasmalemma is the use of inhibitors containing the aliphatic substitution group. Acylic derivates of substrates might be effective nonpenetrating inhibitors of most transporters. The suggested method of rate measurement of oxidating substrate transport [28] might be considered as a universal approach.

Earlier the plasma membrane of *S. cerevisiae* was thought to lack a protein-mediated system of C_4-dicarboxylate transport [15, 56, 57]. At pH 3.0, only L-malate transport mediated by diffusion of its uncharged form was shown [56]. Transport of succinate in *S. cerevisiae* we have determined using the endogenous system of succinate oxidation by mitochondria (Fig.2) as a coupled system to determine the rate of the substrate entrance into the cells [25, 27].

At neutral pH the K_m value of the mitochondrial "succinate oxidase" was fivefold less than that of the cellular "succinate oxidase". O-Palmitoyl-L-malate completely inhibited cell respiration in the presence of succinate but not glucose or pyruvate. We have earlier found that higher aliphatic derivatives of C_4-dicarboxylates are effective inhibitors of the liver mitochondrial dicarboxylate transporter [59]. O-palmitoyl-L-malate does not penetrate across the plasma membrane of *S. cerevisiae* at pH 5.5. It completely inhibited cell respiration in the presence of succinate but not glucose or pyruvate [59]. Under these conditions, it was expected to influence proteins only of plasma membrane. The linear inhibition in Dickson plots indicates that the rate of succinate oxidation is limited by its transport across the plasmalemma. O-Palmitoyl-L-malate and L-malate were competitive inhibitors (the K_i values were 6.6 ± 1.3 μM and 17.5 ± 1.1 mM, respectively) [25]. The malonate derivative, 2-undecyl malonate, also competitively inhibited the rate of succinate transport (K_i = 7.8 ± 1.2 μM) [25]. Both O-palmitoyl-L-malate and 2-undecyl malonate had no effect on the endogenous respiration of the cells, increased the K_m value of the yeast cell "succinate oxidase". At the same time, these inhibitors didn′t change the maximal rate of the reaction (V_{max} =12.4 nmol/min per mg dry weight and K_m = 8.2 mM in the absence of the inhibitor)[25]. It seems that the active site of the plasma membrane dicarboxylate transporter binds not only L-malate but also malonate. The competition of these inhibitors suggested their interaction with the same point of the substrate binding in the active site of the transporter.

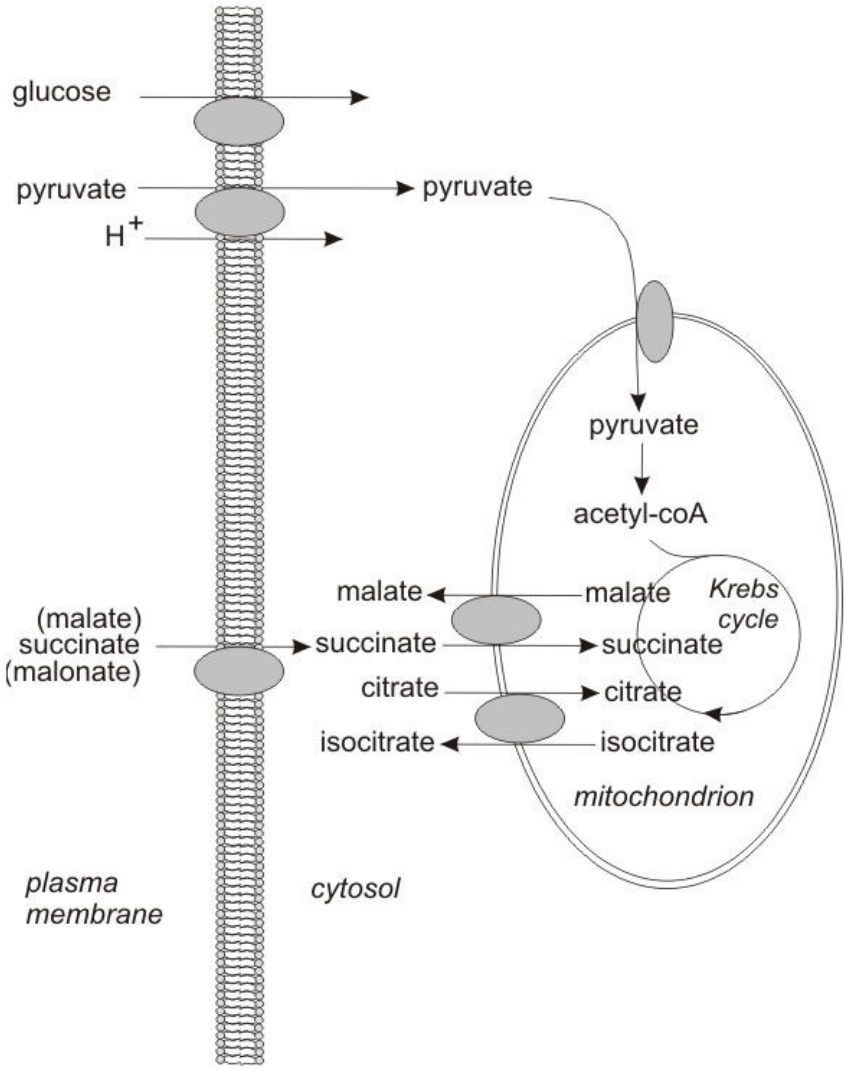

Fig. 2 Oxidation of substrates of plasma membrane dicarboxylate transporter in *S. cerevisiae* (scheme) [27].

Studies on isolated cells are preferable because in this case possible changes in the dicarboxylate transporter properties associated with its reconstruction into liposomes can be prevented, as shown for the tricarboxylate [60] and adenylate [61] mitochondrial transporters. We chosed conditions suitable for measurement of the transport of dicarboxylates (succinate and malonate) across *S. cerevisiae* plasma membrane, when the diffusion of protonated, i.e., uncharged dicarboxylates was absent (pH 5.5 and higher). We have shown that the yeast cells grown at a low concentration of glucose (in the absence of glucose repression) displayed pronounced "endogenous respiration". This respiration was insensitive to specific inhibitors of mitochondrial succinate dehydrogenase - malonate and thenoyltrifluoroacetone. In our experiments [25] the oxidation of pyruvate, glucose, and acetate by the cells was associated (via the Krebs cycle) with functioning of succinate dehydrogenase (Fig. 2). This was supported by the inhibitory effect of thenoyltrifluoroacetone on the oxidation of each of these substrates. Thus, thenoyltrifluoroacetone inhibited the oxidation of acetate (the dependence on the substrate concentration was hyperbolic) with I_{50} of about 0.2 mM [25]. The inhibition of the succinate oxidation was a sigmoid shaped curve with I_{50} of about 0.8 mM [25]. This indicated that the common for these two processes part of the coupled system of succinate dehydrogenase + "ubiquinol oxidase" failed to limit the oxidation of succinate. And the succinate transport across the plasma membrane of *S. cerevisiae* is likely to be a limiting link for the rate of succinate oxidation by the cells (Fig. 2). It is not coupled with proton transport, but sodium ions are necessary. It has been shown [25] that the respiration of *S. cerevisiae* cells in the presence of succinate is associated with existence of a carrier catalyzing the transport of dicarboxylates (succinate, L-malate and malonate). This was supported by the following data [25]: the cells oxidize succinate (pK_a values are 4.21 and 5.72) at pH 6.5 in the absence of its undissociated form; the oxidation rate dependence on the succinate concentration is a curve with saturation; the K_m values for succinate of the plasmalemma and mitochondrial transporters are different (4.4 ± 1.3 and 0.85 ± 0.173 mM, respectively). As it was shown [45], phosphate anion is not bound with the plasma membrane dicarboxylate transporter and seems to be not transported by it. O-Palmitoyl-L-malate does not

penetrate into the cell and effectively inhibits the transport (K_i = 6.6 ± 1.3 μM) [59]. The increase in the rate of succinate oxidation after aerobic preincubation of the cells at 0°C cannot be explained by appearance of cells with damaged plasma membrane. O-Palmitoyl-L-malate inhibited oxidation of succinate by mitochondria nearly 30-fold stronger than its oxidation by intact cells. The dicarboxylate transport was not observed in the monopotassium medium. "Succinate oxidase" manifested itself in the Na^+containing medium.

5. Lipophilic derivatives of substrates and topography of the active site of transporters

We studied earlier the topography of the active site channel of the rat liver mitochondrial dicarboxylate transporter [23]. The studies were performed using competitive inhibitors, 2-monoalkylmalonates. Changes in the inhibition constants of these compounds ($\Delta K_i = K_i(n) - K_i(n\text{-}1)$) on lengthening by one methylene link characterized the degree of lipophilicity in the region of the terminal methyl group binding. It has been shown that near the substrate-binding site and at the channel exit a small and large lipophilic area, respectively, is located, with a clearly expressed polar region between them [23]. The sizes of these regions were 0.38, no less than 0.88, and 0.50 nm, respectively. The outer semi-channel was of no less than 1.76 nm in length. The thickness of the membrane hydrophobic matrix was taken as 4.0 nm. We suggested that the substrate-binding site of the active site should be located in the middle of the membrane [23]. Such symmetry is a characteristic feature of mitochondrial transporters, antiporters.

2-alkylmalonates and O-acyl-L-malates have been found to competitively inhibit the dicarboxylate transporter of *S. cerevisiae* cells, and the substrate derivatives used did not penetrate across the plasma membrane under our experiment conditions. Comparison of the results of the active site probing using these inhibitors allowed us to investigate specific features of the substrate-binding site and its environment.

Probing of the active site of the transporter has revealed a large lipophilic area stretching between the 0.72 to 2.5 nm from the substrate-binding site [26]. We suggested the existence of a hydrophobic region immediately near the dicarboxylate-binding site (to 0.72 nm) because itaconate inhibited the transport fivefold more effectively than L-malate. Fumarate but not maleate competitively inhibited succinate transport into the cells. It was suggested that the plasma membrane transporter binds the substrate in the *trans*-conformation [26].

Variable lipophilicity has been shown to characterize the exposed into the channel surface of the third transmembrane segment of the *S. cerevisiae* mitochondrial citrate/malate antiporter. The structure of this transporter resembles the tertiary structure of the adenylate transporter [20]. This transporter also has the substrate-binding site exposed into the channel [17]. If the malate and malonate heads of the inhibitor of the plasmalemmal transporter are bound in the same site (it was indirectly confirmed by our data on the comparative efficiency of inhibition by itaconate and L-malate), the plasma membrane channel remains lipophilic at the distance of 2.5 nm from the substrate-binding site, which is equal to the length of O-stearoyl-L-malate. The length of the hydrophobic part of the lipophilic profile was estimated as the difference between the lengths of O-stearoyl-L-malate (2.52 nm) and 2-pentylmalonate (0.75 nm), i.e. the longest and the shortest molecular probes. This difference was 1.74 nm [26].

The well studied plasmalemmal transporters with known tertiary structure [21] are shown to have a single substrate-binding site or the selectivity determining region per molecule. Based on the presence of unit arginine residues in the transmembrane segments, all transporters of C_4-dicarboxylates are supposed to have a single substrate-binding site exposed into the channel. Our approach is promising for positioning this point on the transmembrane segment surface, which is exposed into the channel, relative to arginyls of the substrate-binding site. Plasma membrane transporters are significantly different from mitochondrial transporters the in size, number of transmembrane segments, and functioning mechanisms; only the channel presence in the structure is their common feature. It is reasonable to suggest that this channel is the only place in the transporter's structure where the aliphatic chain of the competitive inhibitor can have a conformation with the minimum energy [23]. The applicability of the proposed approach has been shown for scanning the lipophilic profile of the channel in both groups of transporters.

6. Dicarboxylate transporter is involved in succinate and citrate influx and is modulated by pH and cations

Succinate and citrate transport into yeast cells was studied by measuring substrate oxidation rates in the presence and in the absence of O-palmitoyl-*L*-malate and 2-undecyl malonate [27]. Linearity of the Dickson plot for 2-undecyl malonate suggests that this inhibitor blocked the rate-limiting step upon oxidation of both substrates, which was, most probably, transport of these substrates across the plasma membrane (due to inability of the inhibitor to penetrate into the membrane). In the case of succinate transport, the limiting rate of succinate oxidation depended on pH and increased monotonously from near-zero at pH 4.5 to the maximum level at pH 7.5. Succinate and citrate transport was insensitive to the protonophore FCCP a pH 5.5 and was activated by Na^+ ions and competitively inhibited by 2-undecyl malonate and K^+ ions. 2-Undecyl malonate increased K_m without any effect on the rate of succinate oxidation for both succinate and citrate. The competitive inhibition constants for succinate and citrate transport were comparable with the K_i values calculated from our experimental data (6.7 and 5.7 μM, respectively) [27]. Such substrate specificity is atypical for

fungi. In monosodium media pH increase was accompanied by a decrease of succinate K_m due to higher proportion of the dianionic form of the substrate. The dianionic form of the substrate was most probably, the dominant transported form. This finding is consistent with our data on competitive inhibition of plasmalemmal succinate transport by fumarate (but not maleate) [26]. Such stereospecificity of inhibition usually takes place during binding of the dianionic form of the substrate to the active site of the transporter. It seems that in our conditions citrate is transported in the dianionic form and the dicarboxylate transporter is appropriate to its name. Our data demonstrated with a high degree of probability, that citrate is transported in the dianionic form, succinate and citrate transport is mediated by a common protonophore-insensitive plasmalemmal transporter, which is activated by Na^+ ions at pH 5.5 [27].

Broad substrate specificity is characteristic feature of many known yeast dicarboxylate transporters. But their ability to carry citrate and the effects of cations on transport rate are not documented in the literature [1, 2, 4, 5]. The dicarboxylate transporter described in [25-27] has no analogs among dicarboxylate transporters of fungal plasmalemma. Na^+-dependent plasma membrane dicarboxylate symporters able to transfer citrate were detected in animals [62], plants [63], and bacteria. Cationic modulation of transport activity was established for some antiporters of mammalian zwitterionic amino acids [64]. Some fungi (e.g., *Penicillium simplicissimum* [65]) simultaneously excrete citrate and dicarboxylates. However for this transport it has not been shown the same sensitivity to a common specific inhibitor. Functional characteristics of the transporter studied in our recent works [25, 27, 28] make it possible to suggest mechanisms of its activity. The following mechanisms are known: facilitated diffusion, proton or cation symport, ATP-dependent transport, electroneutral antiport. The proton symport of dicarboxylates is excluded by the fact of insensitivity of this transport to plasmalemma-deenergizing concentrations of the protonophore. This is a strong argument against the hypothesis that this transport is an accessory function of wellknown *S. cerevisiae* plasmalemmal proton symporters effecting the transport of structural analogs of succinate, e.g., ureidosuccinate [66] and aspartate [67]. Succinate oxidation by *S. cerevisiae* cells is insensitive to protonophore concentrations favoring glucose oxidation. An electroneutral antiport mechanism requires commensurate dicarboxylate concentrations on both sides of the plasmalemma. In the case of the dicasrboxylate transporter, they must be commensurate with the K_m value for succinate varying from 1.65 ± 0.02 mM to 7.6 ± 1.2 mM [25]. External media for nonparasitic unicellular organisms should not contain high concentrations of the substrate. Yeast cells, utilizing dicarboxylates as growth substrates, contain high-affinity plasmalemmal transporters. K_m values of *P. tannophilus* [4], *K. marxianus*, and *C. utilis* [2] cells for succinate at pH 5.5 are equal to 0.064, 0.031, and 0.124 mM, respectively. The ability of *S. cerevisiae* cells to oxidize succinate in the presence of nonpenetrating Tris cations, excludes the possibility of cation symport mechanism. Low affinity and broad specificity suggest that the *S. cerevisiae* dicarboxylate transporter unlikely plays the role of the sensor for regulatory metabolites as is described for specific phosphate or glucose transporters with relatively low activity in *S. cerevisiae* cells but high affinity to the substrate [68]. The kinetic characteristics and other properties of the plasma membrane dicarboxylate transporter [25-27] are consistent with the mechanism of facilitated diffusion. In this case, the tested cations can play the role of affinity modulators for independent allosteric centers of the transporter molecule.

Transport of dicarboxylates plays an important role in cell metabolism [29]. Primary structures for transporters with known kinetic mechanism and kinetic transport parameters are of particular interest now. For each studied group of organisms the number of transmembrane segments in the transporter molecule and the substrate specificity do not correlate with a certain transport mechanism - antiport, symport with proton or symport with cation. It is of great scientific interest various perspective methodical approaches allowing association of specific features of structure with transport mechanism for individual transporters, construction of functional hybrid transporters - «protein chimeras», scanning of transporter transmembrane segments with the help of «cystein mutagenesis», study of transporter kinetic parameters with point mutations of essential amino acids, probing the transporter active site with alkyl- and acyl-substrate derivatives for studying «lipophilic profiles» of dicarboxylate transporter channels. It is recommended [29] to use all these approaches to one transporter with small sizes and large substrate specificity.

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