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# Succinate Dehydrogenase of *Saccharomyces cerevisiae* – The Unique Enzyme of TCA Cycle – Current Knowledge and New Perspectives

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## 1. Introduction

Glycolysis is an anabolic pathway common in both aerobic and anaerobic organisms. Sugars and polysaccharides have to be transformed into glucose or one of its phosphorylated derivatives before being processed any further. In the course of degradation, ATP is produced. Pyruvate may be regarded as the preliminary final product of the degradation - in a strictly formal sense - because it is here that the pathway ramifies: pyruvate is hydrated under anaerobic conditions resulting in either lactate (in lactic-acid bacteria) or ethanol (in yeast). If glycolysis results in these final products, it is spoken of fermentation. In the presence of oxygen pyruvate is converted to acetyl-coenzyme A and oxidized to $CO_2$ in the tricarboxylic acid (TCA) cycle (Figure 1). In yeasts, as well in most non-photosynthetic cells, mitochondrial oxidative phosphorylation is the main process of ATP synthesis in aerobic conditions. Aerobic pathways permit the production of 30 to 38 molecules of ATP per one molecule of glucose. Although two molecules of ATP come from glycolysis and two more directly out of the TCA cycle, most of the ATP arises from oxidative phosphorylation.

The main catalytic function of the TCA cycle is to provide reducing equivalents to the respiratory chain through the oxidative decarboxylation of acetyl–CoA (8), but every TCA cycle intermediate is commonly used by other metabolic reactions. The eight enzymes from the TCA cycle are encoded by 15 nuclear genes in *S. cerevisiae* [1]. The first reaction of TCA is catalyzed by citrate synthase (1) and it is the condensation of acetyl–CoA and oxaloacetate resulting in the formation of citrate. The second reaction of the TCA is catalyzed by aconitase (2), leading to the conversion of citrate into isocitrate. Aconitase is located both in mitochondria and in cytosol. The next step of the TCA is the oxidative decarboxylation of isocitrate to α-ketoglutarate (3). There are three known isoenzymes of isocitrate
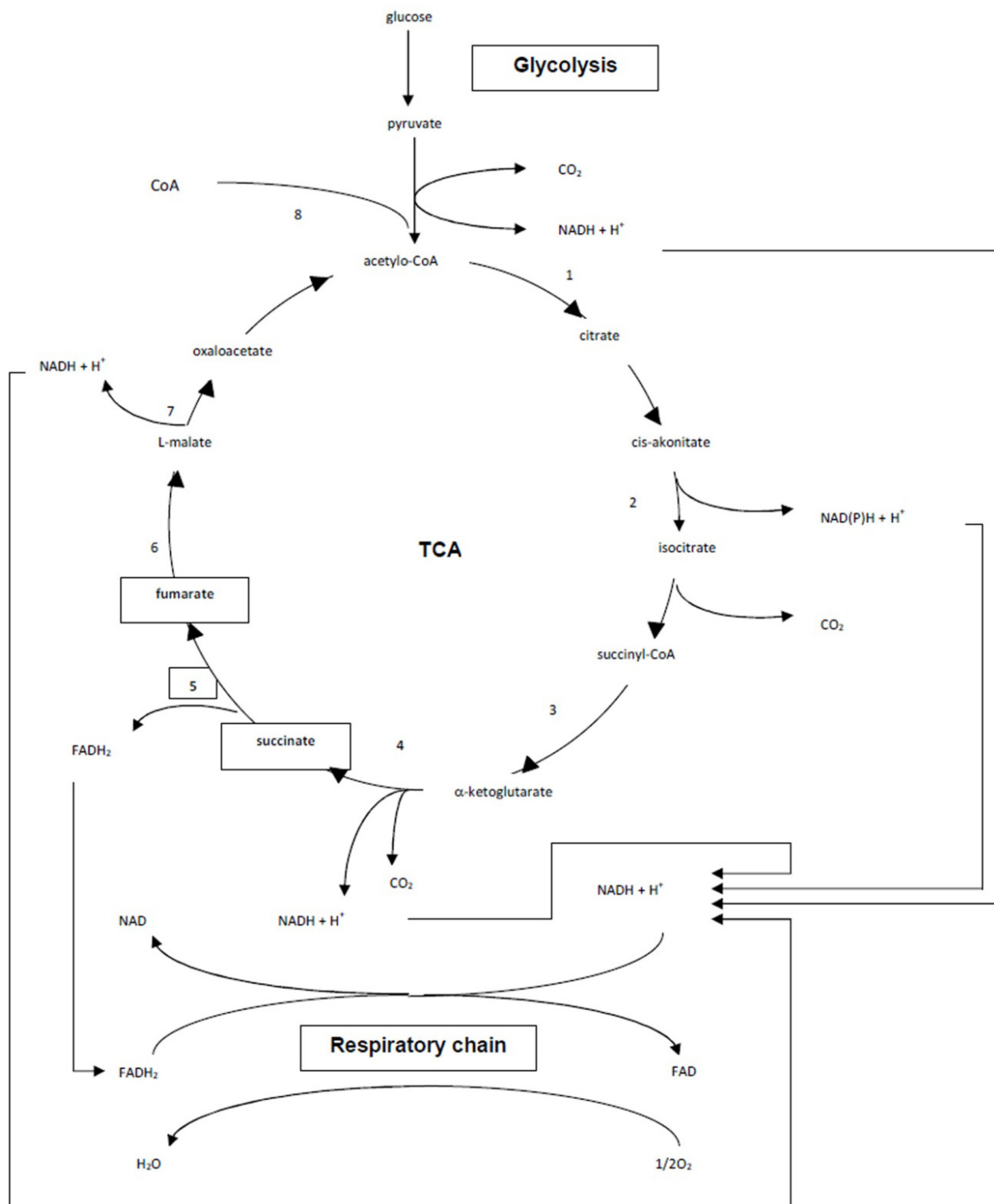
**Figure 1.** TCA cycle.

dehydrogenase, a mitochondrial $NAD^+$-specific one and two $NADP^+$-dependent ones (one mitochondrial and the other cytosolic). A number of pieces of evidence point to the role of mitochondrial $NAD^+$-specific isocitrate dehydrogenase in the regulation of the rate of mitochondrial assembly besides its specific role in the TCA cycle [2]. The formation of

succinate is catalyzed by α-ketoglutarate dehydrogenase (4), which promotes the oxidative decarboxylation of α-ketoglutarate via succinil–CoA to succinate, which is then converted to fumarate by succinate dehydrogenase (5). The next step of the TCA cycle is the conversion of fumarate to malate by the enzyme fumarase (6), which exists as separate cytosolic and mitochondrial forms. There is not yet a clear explanation for the existence of these two forms; however, the localization and distribution of fumarase appears to be unique because there is only one translation product, which is targeted to mitochondria [3]. Malate dehydrogenase (7) catalyzes the last step of the TCA and leads to the oxidation of malate to oxaloacetate. There are three isoenzymes of malate dehydrogenase: a cytosolic, a mitochondrial and a peroxissomal one; however, the mitochondrial one accounts for 90% of malate dehydrogenase activity when glucose is being metabolized [4].

TCA cycle flux appears to be constricted at two steps on the basis of the limited availability of the substrates oxaloacetate and α-ketoglutarate. The genes encoding TCA proteins might also be regulated by glucose levels. In *S. cerevisiae* the depletion of glucose increases 3–10 times the TCA messenger RNAs [5]. Oxygen limitation could also induce a shift in the TCA cycle, which operates as a cycle during aerobic growth [6].

All reactions of glycolysis take place in the cell cytosol. The citric acid cycle and the respiratory chain are located within mitochondrial matrix. In contrast with all of the other enzymes of the TCA cycle, which are soluble proteins found in the mitochondrial matrix, succinate dehydrogenase (SDH) is an integral membrane protein tightly associated with the inner mitochondrial membrane [7]. Membrane-bound succinate dehydrogenase (EC 1.3.99.1) or succinate-coenzyme Q reductase or Complex II, is present in all aerobic cells. It was discovered by Thunberg in 1909. Now we know that SDH has several particularly interesting properties: (1) it is a membrane-bound dehydrogenase linked to the respiratory chain and a member of the tricarboxylic acid (TCA) cycle; (2) its activity is modulated by several activators and inhibitors; and (3) it is a complex enzyme, and covalently bound flavin adenine dinucleotide (FAD) [8].

## 2. SDH structure

SDH is a heterotetramer composed of flavoprotein (Fp) of about 70 kDa, an iron-sulfur protein (Ip) of about 30 kDa, and two hydrophobic anchoring subunits of 7-17 kDa. The Fp contains the active site and the unusual cofactor, an $8\alpha$-$N$(3)-histidyl-FAD linked at a conserved histidine residue (Figure 2).

FAD attachment is stimulated by, but not dependent upon, the presence of the iron-sulfur subunit and citric acid cycle intermediates such as succinate, malate, or fumarate [9].

The Ip subunit contains three different iron-sulfur clusters: [2Fe-2S], [3Fe-4S], and [4Fe-4S] (Figure 3).

These clusters are coordinated by conserved cysteine residues: [2Fe-2S] (Cys-67, Cys-72, Cys-75, Cys-87); [4Fe-4S] (Cys-159, Cys-162, Cys-165, Cys-226) and [3Fe-4S] (Cys-169, Cys-216, Cys-222) [10].
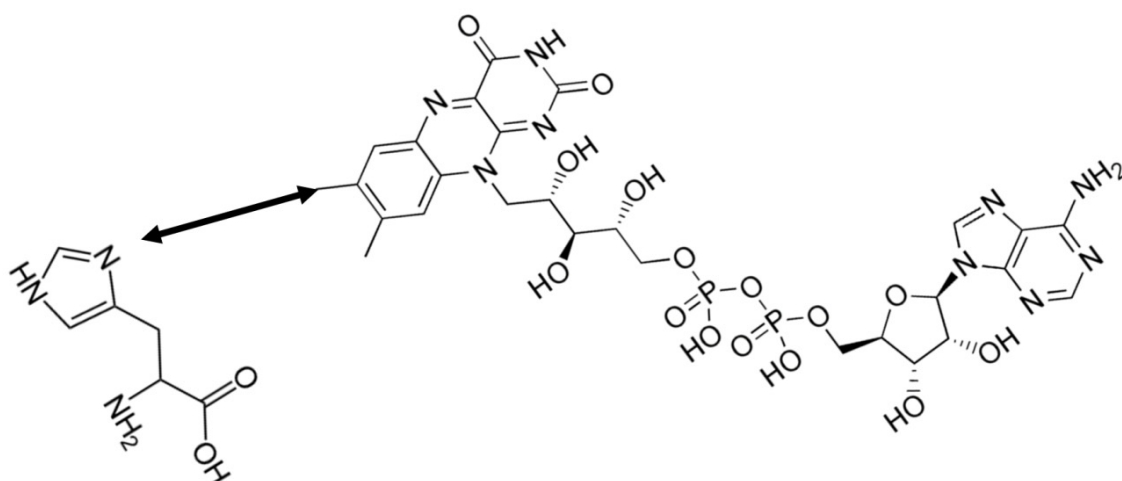
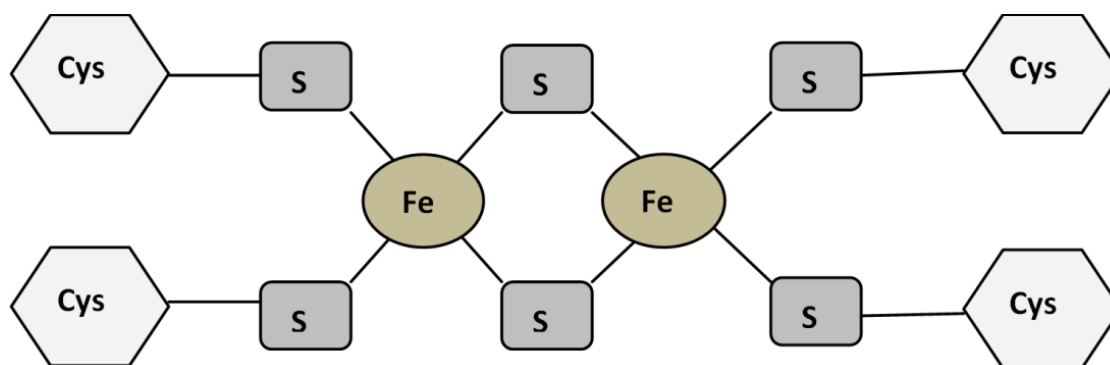**Figure 2.** The covalent bond between FAD and succinate dehydrogenase.



**Figure 3.** The 2Fe-2S cluster of succinate dehydrogenase [9].

The hydrophobic anchoring subunits are integral membrane proteins and interact with quinone substrates. The yeast and mammalian SDH also contains a *b*-type heme. Oyedotun et al. [11] demonstrated the presence of an amount of cytochrome b₅₆₂ steichiometric to covalent FAD. Together, the Fp and Ip form a catalytic dimer that is attached to the membrane by the anchoring subunits, thereby composing the holoenzyme. In yeast, the SDH Fp, Ip, and two anchoring subunits are encoded by the nuclear genes, *SDH1*, *SDH2*, *SDH3*, and *SDH4*, respectively, which have all been cloned and sequenced. The SDH subunits are translated in the cytoplasm, targeted to mitochondria by cleavable amino-terminal presequences, translocated across both mitochondrial membranes, and finally assembled with each other and their respective co-factors into a functional complex [10].

The quaternary structure model of the SDH for different cells, e.g. *S. cerevisiae* and mammalian, was described in study conducted by Oyedotun and Lemire [10] and Yankovskaya et al. [12]. First subunit of SDH provides the binding site for the oxidation of succinate. The side chains Thr254, His354, and Arg399 stabilize the molecule while FAD oxidizes and carries the electrons to the first of the iron-sulfur [2Fe-2S] clusters. Whereas, ubiquinone binding site is located is in a gap composed of three SDH subunits. Ubiquinone is stabilized by the side chains of His207 of second subunit, Ser27 and Arg31 of third subunit C, and Tyr83 of fourth subunit. The quinine ring is surrounded by Ile28 of third subunit and

Pro160 of second subunit B. These residues, along with Il209, Trp163, and Trp164 of second subunit B, and Ser27 (C atom) of third subunit, form the hydrophobic environment of the quinine binding pocket. The succinate binding site and ubiquinone binding site are connected by a chain of redox centres including FAD and the iron-sulfur clusters. This chain extends over 40 Å through the enzyme monomer with an edge-to-edge distance between the centres is less that 14 Å limit for physiological electron transfer [8, 11].

In the place for heme *b*, the N 2 atom of Sdh3p His106 and the S atom of Sdh4p Cys78 are correctly oriented to form coordinating bonds with the central iron atom of the heme. The distance between the iron atom and the N 2 atom is 2.21 Å, but  the S atom of Cys78, the putative second heme axial ligand, is 2.96 Å away from the heme Fe. UQ can be docked into two spatially separated sites with an edge-to-edge distance of 25.4 Å. The new amino acid residues that may determine the structural or catalytic properties of each of the two quinone binding sites were identified. The model also provided insight into the unusual use of a cysteine (Sdh4p Cys78) as the second heme ligand instead of the histidine residues [10].

## 3. SDH function and regulation

Succinate dehydrogenase is a key enzyme in intermediary metabolism and aerobic energy production in living cells. This enzymes catalyses the oxidation of succinate into fumarate in the Krebs cycle (1), derived electrons being fed to the respiratory chain complex III to reduce oxygen and form water (2). This builds up an electrochemical gradient across the mitochondrial inner membrane allowing for the synthesis of ATP. Alternatively, electrons can be diverted to reduce the ubiquinone pool (UQ pool) and provide reducing equivalents necessary to reduce superoxide anions originating either from an exogenous source or from the respiratory chain itself (3) [13] (Figure 4).
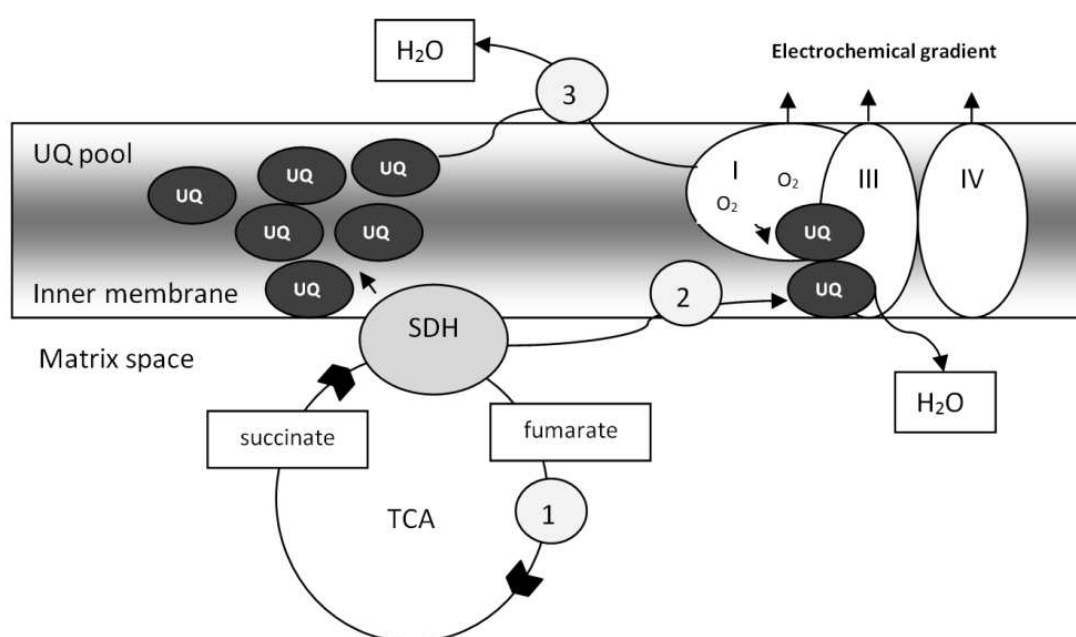


**Figure 4.** The functions of the succinate dehydrogenase in the mitochondria [13].

In the reaction of oxidation of succinate to fumarate, two hydrogen atoms are removed from substrate by flavin adenine dinucleotide (FAD), a prosthetic group that is tightly attached to succinate dehydrogenase (Figure 5).
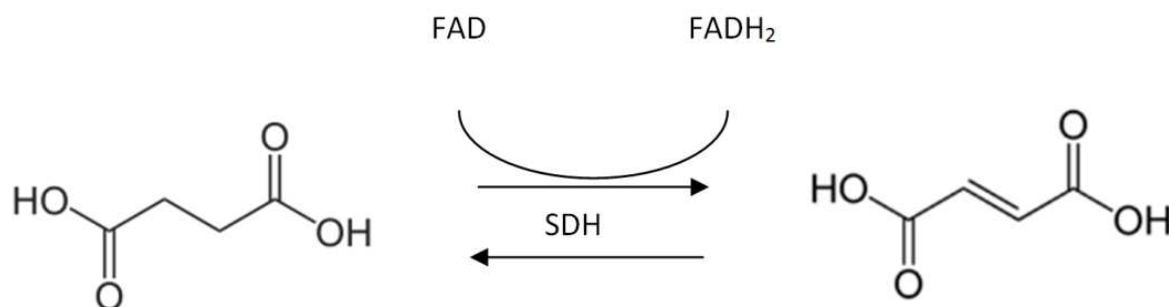


**Figure 5.** The succinate dehydrogenase reaction.

Two electrons from the reduced SDH-FADH2 complex are then transferred to ubiquinone (Q), a soluble component of the electron transport system complex II. Ubiquinone is then reduced to ubiquinol ( QH2).

Flavin adenine dinucleotide (FAD) is an essential cofactor for SDH enzyme. The generation of adenosine triphosphate (ATP) in mitochondria is coupled to the oxidation of nicotinamide adenine dinucleotide (NADH) and FADH2 and reduction of oxygen to water within the respiratory chain and a three-dimensional structure of the mitochondrial respiratory membrane protein complex II. FAD attachment is stimulated by, but not dependent upon, the presence of the iron-sulfur subunit and citric acid cycle intermediates such as succinate, malate, or fumarate [9]. The substrate analog malonate is a competitive inhibitor of the succinate dehydrogenase complex. Malonate, like succinate, is a dicarboxylate that binds to cationic amino acid residues in the active site of the succinate dehydrogenase complex. However, malonate cannot undergo oxidation because it lacks the -CH2 - CH2- group necessary for dehydration. To study the effects of a competitive inhibitior on the activity of succinate dehydrogenase, malonate will be added to a reaction mixture; malonate is sufficiently different from succinate that it cannot de dehydrogenated, i.e. malonate is not metabolized [8, 14].

SDH is a difficult enzyme to extract from respiratory membrane whilst still retaining its *in vivo* properties. Most of the extraction procedures used in early work were rather drastic and yielded soluble preparations of rather dubious integrity. However, the recent introduction of a more gentle method, involving disruption of the membrane with chemotropic agents, has yielded an active and nearly homogeneous enzyme of relatively low molecular weight (97,000). This enzyme can be separated by freezing and thawing into two inactive subunits. One of these, an iron sulphur flavoprotein of molecular weight 70,000, contains one mole of FAD and four moles each of iron and labile sulphide per mole of protein; The other, an iron-sulphur protein of molecular weight 27,000, also contains four moles each of iron and labile sulphide. It was determined that the large subunit of SDH is essential for catalytic activity, but the function of the small subunit, be it catalytic or regulatory [8].

Electronic paramagnetic resonance measurements of SDH components indicated that at least three separate centres are present. The S-3 centre (E'o = +60 mV) is a high potential Fe-S protein and is probably identical with the 4Fe-rS centre of low molecular weight subunit. Centres S-1 and S-2 (E'o = +15 mV and -260 mV respectively), are of the 2Fe-2S ferredoxin type and are probably associated with a larger, flavin containing subunit. Thus, electron transfer from succinate to ubiquinone probably occurs in the sequence: FAD, S-1, S-3. The redox potential of S-2 is rather too low to allow this centre to be catalytically active in the forward direction [8,15].

The catalytic activity of succinate dehydrogenase is modulated both by post translational phosphorylation/acetylation and active site inhibition. For example, phosphorylation of the Sdh1 subunit leads to attenuate activity of SDH. The activity of this enzyme may be also modulated by Krebs cycle intermediates including oxaloacetate or malonate which are strong inhibitors. Mechanisms of inhibition by these compounds differ significantly because oxaloacetate, a competitive inhibitor of succinate dehydrogenase, bounds with a sulfhydryl group of the enzyme to abolish the enzyme activity [16]. It is known that SDH is sensitive to different thiol-binding reagents. Inhibition of the enzyme by these kinds of reagents resulted from the modification of a sulfhydryl group located at the active site. This thiol, although not essential for substrate binding or catalysis, could influence the binding of dicarboxylates, probably by steric hindrance when a larger group or a charged group were attached to it. The inhibition of SDH by histidine specific reagents was also reported, and the participation of an imidazole ring in the initial step of succinate oxidation was suggested. The inactivation of SDH by phenylglyoxal and 2,3 –butanedione showed the presence of an arginine-residues  that interacts with dicarboxylate to form the primary enzyme-substrate complex [17].

SDH is not only known to catalyse a unique reaction, which requires the participation of its four subunits, but deleterious mutations in any of the SDH genes should invariably result in a decreased SDH activity. Therefore, the striking phenotypic differences associated with mutations in the four subunits raise puzzling questions. SDH also plays a specific role in the maintenance of the mitochondrial UQ pool reduction. Ubiquinone, beside its function as an electron carrier mediating electron transfer, is admittedly working as a powerful antioxidant in biological membranes. Then, only a portion of the UQ pool may be actually involved in electron transfer depending on dehydrogenases involved. Accordingly, the measurable redox status of the UQ pool should result from the reducing activity of the different dehydrogenases, the oxidising activity of complex III and the kinetic equilibrium in the pool. The UQ pool therefore represents an electron sink and, when reduced, an antioxidant reservoir in the mitochondrial inner membrane. However, UQ is a double-faced compound, possibly working as either an antioxidant when fully reduced to ubiquinol, or a pro-oxidant when semi-reduced to the unstable ubisemiquinone form. Possibly together with reduced cytochrome b, semi-reduced quinones constitute the prominent source of superoxides. Finally, when defective, the abnormal amount of superoxides can be produced, e.g.  flavin radicals of complex I. Delivering electrons for the full reduction of UQ to UQH2 might then be of a tremendous importance for the control of oxygen toxicity in the mitochondria.

Therefore, the SDH, thanks to its unique redox properties, may be a key enzyme to control UQ pool redox poise under these conditions [13].

Also mutations in genes encoding SDH subunits lead to reduced activity of SDH enzyme. The yeast cells disrupted in *SDH2* (*sdh2Δ*) showed dramatically accumulate succinate resulting in inhibition of at least two $\alpha$-ketoglutarate dependent enzymes that generate succinate as a by-product. Disruption of complex II activity should alter TCA cycle metabolite levels in the mitochondrial matrix. It was found that neither *sdh1Δ*, nor s*dh2Δ* cells have measurable SDH activity. The succinate accumulates to 8-fold higher levels in *sdh2Δ* cells relative to wild-type cells. Furthermore, complex II + III activity was completely abolished in both SDH mutants without a corresponding compensation in NADH dehydrogenase activity. As a result, complex IV activity was decreased in the SDH mutants [18] (Figure 6).
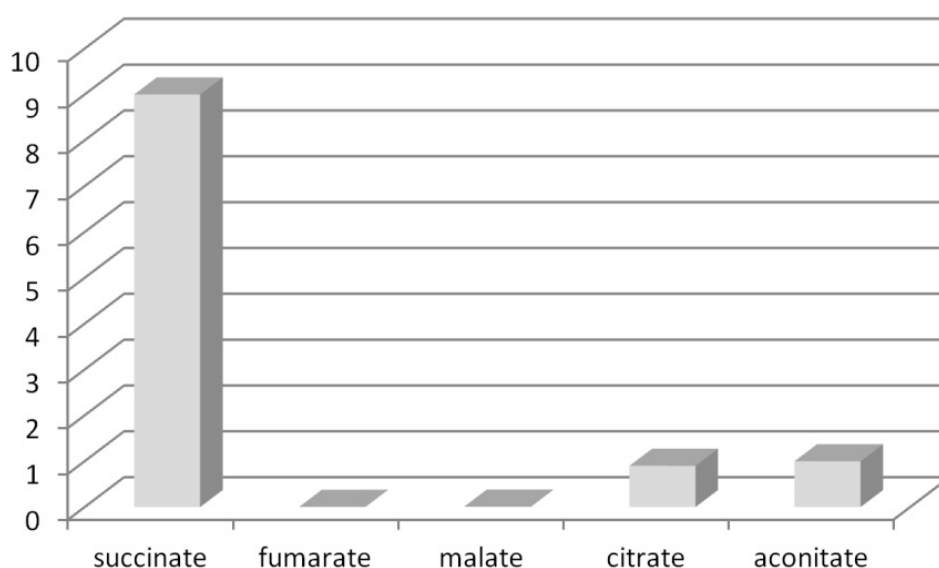


**Figure 6.** Relative concentration of main metabolites of TCA cycle for *sdh1Δ*, nor s*dh2Δ* yeast cells [18].

## 4. SDH activity assay

The succinate is the most efficient energy source, so the SDH activity assay can be an important method for measurement of the yeast vitality in scope to control, e.g. different fermentation processes [19]. SDH activities can be measured *in vitro* in cell lysates or in mitochondrial fraction as well as *in situ* in individual cells. Since SDH is bound to the inner membrane, it is easily isolated along with the mitochondria by different techniques: sucrose density gradient ultracentrifugation, free-flow electrophoresis or a commercially available kit-based method [20]. The mitochondrial fraction is the source of the enzyme. Since none of the key components can be measured directly, the reaction succinate $\rightarrow$ fumarate is measured by monitoring the reduction of an artificial electron acceptor. To use an artificial electron acceptor, the normal path of electrons through the mitochondrial electron transport system must be blocked. This is accomplished by adding either sodium azide or potassium

cyanide to the reaction mixture. These poisons inhibit the transfer of electrons from cytochrome a3 to the final electron acceptor, oxygen, thus electrons cannot be passed along by the preceding cytochromes and coenzyme Q. Instead, the electrons from SDH-FADH2 can be picked up by an artificial electron acceptor, such as the dye 2,6dichlorophenolindophenol (DCIP). The reduction of DCIP can be followed spectrophotometrically since the oxidized form of the dye is blue and the reduced form is colorless. This reaction can be summarized as

$$\text{SDH-FADH2} + \text{DCIP}_{oxid.} \rightarrow \text{SDH-FAD} + \text{DCIP}_{red.} + 2\text{H}^+$$
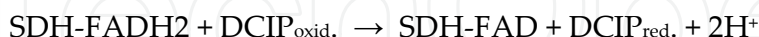
The change in absorbance, measured at 600 nm, can be used to follow the reaction over time [21]. To use an artificial electron acceptor, the normal path of electrons in the electron transport chain must be blocked. This is accomplished by adding either potassium cyanide or sodium azide to the reaction mixture. The rate of the disappearance of the blue color is proportional to the concentration of enzyme. The change in absorbance of the mixture is measured as a function of time and the enzyme concentration is determined from these data. Enzymatic reactions in yeasts are usually studied in cell-free extracts which requires disruption of cells and as consequence, inactivation of particular enzymes often can be observed. Generally we can conclud that determination of SDH enzyme activity has proved to be a difficult enzyme to extract from respiratory membrane whilst still retaining its *in vivo* properties. Most of the described extraction procedures were rather drastic and yielded soluble preparations of rather dubious integrity [8].

In recent years quantitative histochemical procedures has been proved to be a powerful research tool, especially in microphotometric assessment *in situ* of the specific activity of dehydrogenases in individual cells. These assays are simple and valid alternative to conventional biochemical techniques. Methods *in situ* can provide the cellular resolution necessary to determine enzyme-specific activities not only in whole cell preparations but also in distinct subcellular compartments [19].

Reduction of various tetrazolium salts by dehydrogenases of metabolically active cells leads to production of highly colored end products – formazans (Figure 7). The history of the tetrazolium salts and formazans goes back 100 years, to when Friese (1875) reacted benzene diazonium nitrate with nitromethane, to produce a cherry-red "Neue Verbindung". This was the first formazan. Nineteen years later, Von Pechmann and Runge (1894) oxidised a formazan to produce the first tetrazolium salt [21].
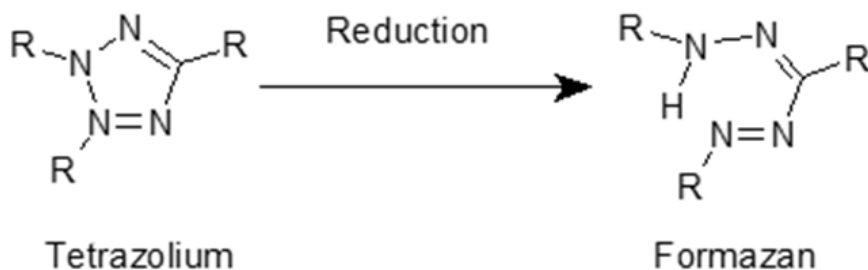


**Figure 7.** Tetrazolium salt and its coloured formazan.

Many hundreds of tetrazolium salts and formazans were prepared in the following years, but only a handful have found applications in biological research.There is a wide range of tetrazolium salts commonly used in the field of microbiology from the classical ones to the new generation of its derivatives. Among them are: blue tetrazolium chloride (BT), 2,3,5-triphenyl tetrazolium chloride (TTC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT), 4-[3-(4-idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST1), 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) or 2,2'-dibenzothiazolyl-5,5'-[4-di(2-sulfoethyl)carbamoylphenyl]-3,3'-(3,3'-dimethoxy-4,4' biphenyl) ditetrazolium, disodium salt (WST-5) [19, 22, 23].

In the case of enzymatic reaction conducted *in situ* the plasma membrane forms a barrier with low degree of penetration. Therefore, cell permeabilization, e.g. by digitonin, is recommended as an alternative method for the study of intracellular enzyme activities. According to the results obtained by Berlowska et al. [23] digitonin was effective in membrane permeabilization without negative influence on cell morphology. After digitonin treatment, the visible formazan crystals were observed inside the yeast cells, but not outside them (Figures 8-10 A, B).

The formazan products are water-insoluble, but readily diffuses out of yeast cells after solubilization in DMSO. Good correlation ($R^2$=0,97) between BTf absorbance intensity after DMSO extraction and number of yeast cells was seen. Linear correlation was observed in the concentration range of yeast cells from $9\times10^7$ to$5\times10^8$ per sample. For yeast cell concentrations below $1\times10^7$ per sample the formazan color intensity signals were too low to detect with good precision. The results obtained for SDH activity were in good agreement
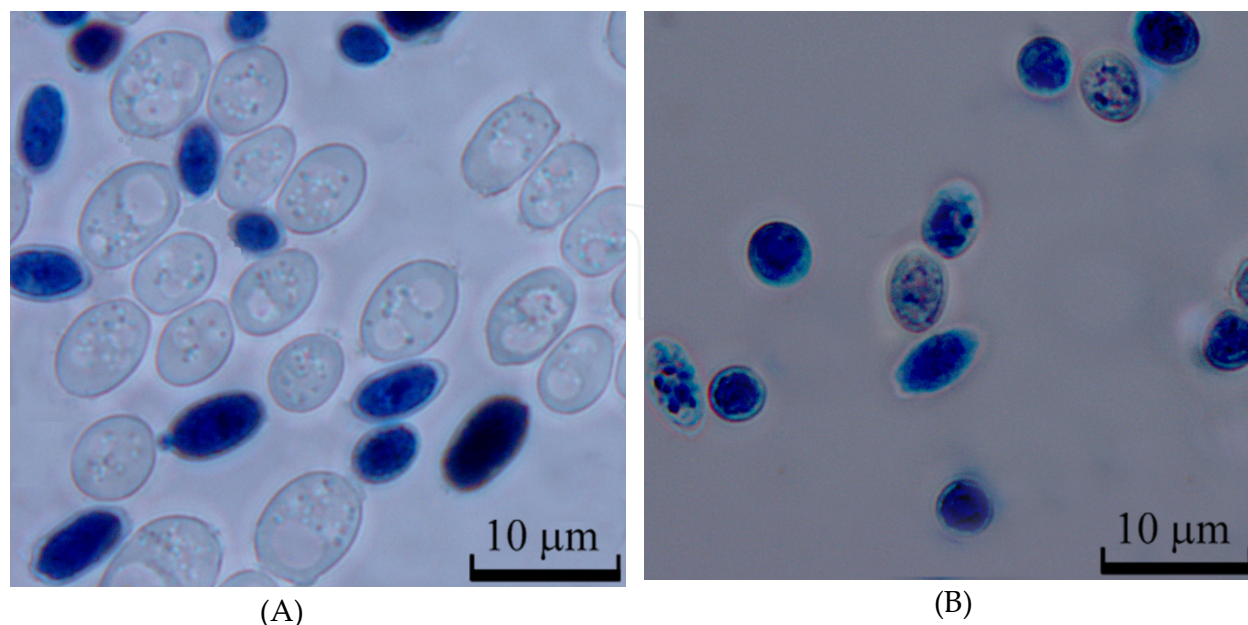


(A)                                                        (B)

**Figure 8.** Yeast cells after reaction with blue tetrazolium chloride (BT). A – without permeabilization; B – with permeabilization by 0.05% digitonin. Images of light microscopy.
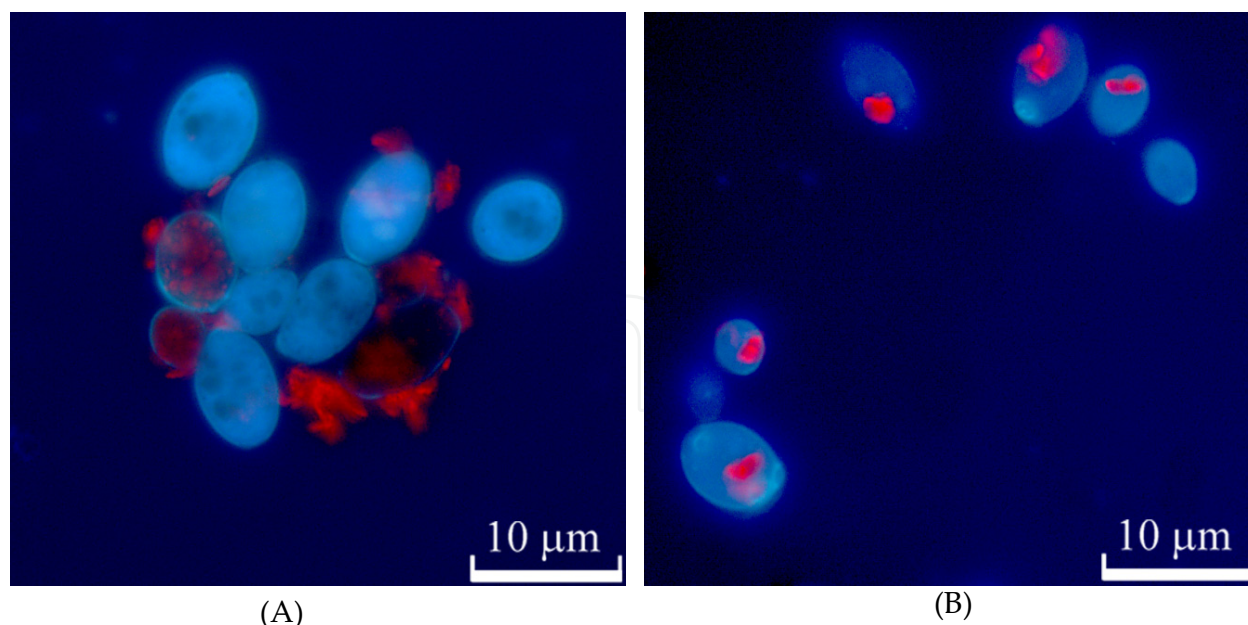
**Figure 9.** Yeast cells after reaction with 2,3,5-triphenyl tetrazolium chloride (TTC). A – without permeabilization; B – with permeabilization by 0.05% digitonin. Images of fluorescence microscopy.
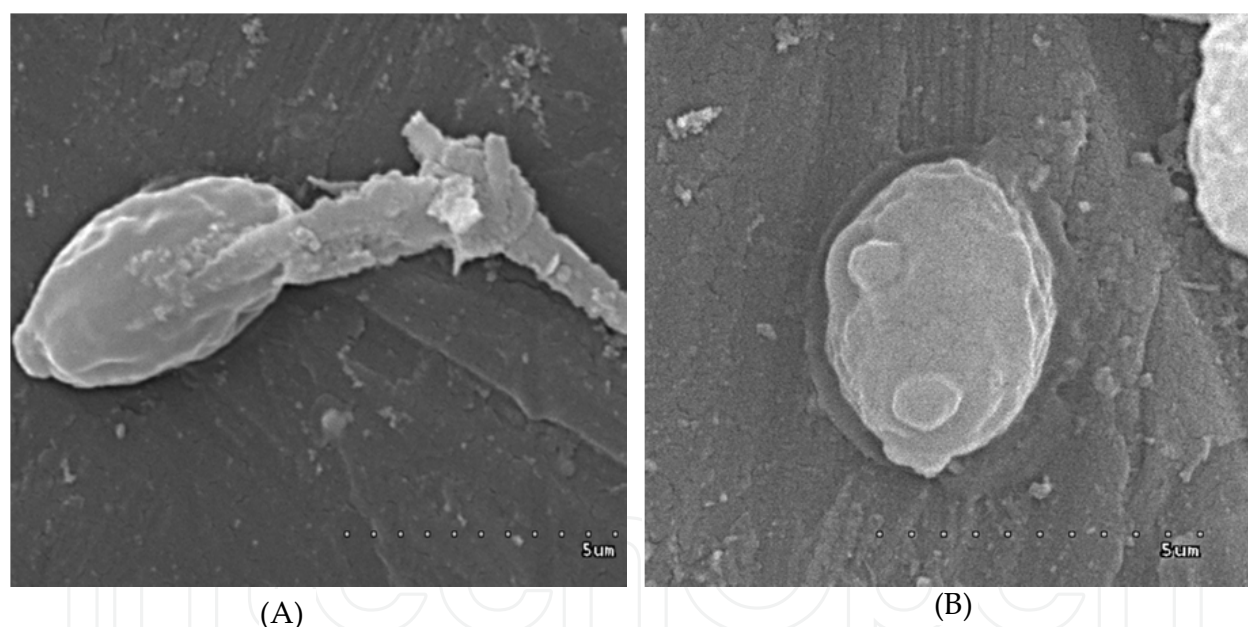


**Figure 10.** Yeast cell after reaction with 2,3,5-triphenyl tetrazolium chloride (TTC). A – without permeabilization; B – with permeabilization by 0.05% digitonin. Images of scanning microscopy.

with that of ATP content in yeast cells. Significant decreasing of succinate dehydrogenase activity and ATP content were observed during aging of tested yeast strains [19, 23].

## 5. The role of SDH in human disease

*Saccharomyces cerevisiae* is a simple eukaryotic organism, with a complete genome sequence. Many genetic tools that have been created during these years, including the complete

collection of gene deletions and a considerable number of mechanisms and pathways existing in higher eukaryotes was first studied and described in yeast. Moreover, about 40% of human genes whose mutations lead to diseases have an orthologue in yeast and genomic screens have been extended to mitochondrial diseases. The study of mitochondrial functions and dysfunction is of special interest in yeast because it is in this organism that mitochondrial genetics and recombination have been discovered and that nucleomitochondrial interactions have been studied in-depth. There are also specific reasons for choosing *S. cerevisiae* for mitochondrial studies. This organism is petite-positive, which can successfully grow in the absence of oxygen. Therefore it can lose its mitochondrial genome provided it is supplied with a substrate for fermentation. Consequently, all mutations of the mitochondrial genome can be studied without cell lethality. The frequency of homologous recombination is very high (1% recombination is considered to correspond to about 100 bp in the mitochondrial genome). It is genetically easy to transfer mitochondria from one nuclear genetic background to another via karyogamy. Additionally, mitochondria can be transformed making in vitro mutation analysis possible. The richness and ease of yeast molecular genetics opens big opportunities, and even the major difference existing between human and yeast mitochondrial genomes, i.e. the predominant heteroplasmy of human and the homoplasmy of yeast, can result in the easier definition of the pathogenic mutations. To review mitochondrial diseases may be a very difficult task because the definition might include different kinds of metabolic disorders or degenerative syndromes [24]. Moreover, some important aspects have been extensively reviewed and the reader might refer to very good recent articles by DiMauro and Garone [25] for historical aspects, by Wallace et al. [26, 27] for bioenergetics, by Spinazzola and Zeviani [28] for nucleo–mitochondrial intergenomic cross-talk. The previous review by Schwimmer et al. [29] was given an important outline of yeast models of mitochondrial diseases. SDH in yeast and human are very similar. They are composed of four subunits (SDHA-D, like SDH1-4 in yeast), all encoded by nuclear genes.

In the last ten years, deficiencies in TCA cycle enzymes have been shown to cause a wide spectrum of human diseases. For instance, mutation in the gene encoding fumarase is a rare cause of encephalomyopathy and a far more common cause of leiomyomas of the skin and uterus and of renal cancer (Table 1).

The TCA path dysfunction may also result from concurrent impairments in several steps of the cycle. The combined deficiencies in SDH and aconitase was observed in Friedreich's ataxia [22, 39]. Measures in autopsied brains from Alzheimer's Disease (AD) patients reveal a decrease in the activity of $\alpha$-ketoglutarate dehydrogenase complex (KGDHC) and an increase in malate dehydrogenase (MDH) activity [33]. The ratios between TCA enzymes are consistent for each mammalian tissues presumably reflecting their metabolic demand. Consequently, in addition to the determination of residual absolute activities, estimation of ratios between enzyme activities is an effective means of detecting partial but potentially harmful deficiencies. When used to assess respiratory chain activities, this approach enabled the identification of several gene mutations, even in patients with partial respiratory chain deficiencies. At present, TCA enzyme activities are measured using a series of independent

| Enzyme | Clinical presentation | References |
|---|---|---|
| Fumarase | Progressive encephalopathy Hereditary leiomyomatosis and renal cell cancer | [30] [31] |
| Malate dehydrogenase | Alzheimer's disease | [32, 33] |
| Citrate synthase | No disease identified so far | |
| Aconitase | No disease identified so far | |
| Isocitrate dehydrogenase | Low-grade gliomas | [34] |
| α-Ketoglutarate dehydrogenase | Congenital lactic acidosis | [35] |
| Succinyl-CoA ligase | Encephalomyopathy with mtDNA depletion | [36] |
| Succinate dehydrogenase | Encephalopathy (Leigh syndrome) Pheochromocytoma and paraganglioma | [37] [38] |

**Table 1.** Primary deficiencies in TCA cycle enzymes in humans [22].

assays that are both laborious and time-consuming. The limited set of assays allowing both measurement of all TCA enzyme activities and detection of abnormalities in enzyme activity ratios were developed. These assays were used successfully to detect severe and partial isolated deficiencies in several TCA enzymes. The first assay measures succinyl-CoA ligase, SDH, glutamate dehydrogenase (GDH), fumarase, and malate dehydrogenase. This assay was performed in medium containing 50 mM KH2PO4 (pH 7.2) and 1 mg/ml BSA. The reduction of DCPIP was measured using two wavelengths (600 nm and 750 nm) with various substrates and the electron acceptors decylubiquinone and phenazine methosulfate. The second assay measured α-ketoglutarate dehydrogenase, aconitase, and isocitrate dehydrogenase activities. The pyridine nucleotide (NAD+/NADP+) reduction is measured with various substrates using wavelengths of 340 nm and 380 nm. In the third assay, citrate synthase was measured by monitoring dithionitrobenzene (DTNB; Ellman's reagent) reduction at wavelengths of 412 nm and 600 nm [22].

The deleterious mutations in any of the SDH genes should invariably result in a decreased SDH activity [13]. Hence, SDH 'inactivation' induces abnormal stimulation of the hypoxia-angiogenesis pathway. Therefore, the striking phenotypic differences associated with mutations in the four subunits raise puzzling questions. It has been reported that inherited deficiencies of SDH associated with presence of mutant protein Fp are always associated with relatively high residual activities, ranging from 25-50% of control mean values. As a comparison, less than 5% residual activity is frequently measured in patients with severe defect of complex IV or complex I. The mutations in any of the SDH cause the complex II to fully disassemble. When complex II is absent, it can be disregarded as a source of additional superoxide production. Thus, the superoxide overproduction would lead to tumour formation that should be ascribed to the decreased ability of the SDH to adequately reduce the Q pool, a necessary condition to resist oxidative stress [8].

Ubiquinone, beside its function in the respiratory chain as an electron carrier mediating electron transfer between the various dehydrogenases and the cytochrome path, is working as a powerful antioxidant in biological membranes [13]. It is possibly for this exact reason in much larger amounts compared to other electron carriers of the respiratory chain, including the sum of the dehydrogenases. When it is defective, the respiratory chain can produce an abnormal amount of superoxides involving additional respiratory chain components such as flavin radicals of complex I. Delivering electrons for the full reduction of Q to QH2 might then be of a tremendous importance for the control of oxygen toxicity in the mitochondria. Therefore, the SDH is a key enzyme to control Q pool redox poise under these conditions, due to its unique redox properties [8].

Iron-sulfur (Fe-S) proteins facilitate multiple functions, including redox activity, enzymatic function, and maintenance of structural integrity. More than 20 proteins are involved in the biosynthesis of iron-sulfur clusters in eukaryotes. Defective Fe-S cluster synthesis not only affects activities of many iron-sulfur enzymes, such as aconitase and succinate dehydrogenase, but also alters the regulation of cellular iron homeostasis, causing both mitochondrial iron overload and cytosolic iron deficiency. Fe-S cluster biogenesis takes place essentially in every tissue of humans, and products of human disease genes have important roles in the process [40].

Succinate is an oxygen sensor in the cell and can help turn on specific pathways that stimulate cells to grow in a low-oxygen environment (hypoxia). In particular, succinate stabilizes a protein called hypoxia-inducible factor (HIF) by preventing a reaction that would allow HIF to be broken down. HIF controls several important genes involved in cell division and the formation of new blood vessels in a hypoxic environment. Mutations in genes encoding SDH subunits have been linked to severe encephalopathy and, more recently, to familial paraganglioma (PGL) and pheochromocytoma (PHEO) (PHEO: adrenal gland PGL). Isolated complex II deficiency is a very rare condition, occurring in approximately 2–4% of all respiratory chain enzyme deficiencies. At least three mutations in the SDH genes have been identified in people with PGL or PHEO, which are noncancerous (benign) tumors associated with the nervous system. *SDHB-D* gene mutations are seen most commonly in people with PGL, but they were found in people with PHEO. However, a single mutation in the *SDHA* gene increases the risk that an individual will develop the condition, and additional mutation that deletes the normal copy of the gene is needed to cause tumor formation. This second mutation, called a somatic mutation, is acquired during a person's lifetime and is present only in tumor cells. The SDH genes mutations associated with nonsyndromic PGL or PHEO change single protein building blocks (amino acids) in the SDH protein sequence or result in a shortened protein. As a result, there is little or no SDH enzyme activity. Because the mutated SDH enzyme cannot convert succinate to fumarate, succinate accumulates in the cell. The excess succinate abnormally stabilizes HIF, which also builds up in cells. Excess HIF stimulates cells to divide and triggers the production of blood vessels when they are not needed. Rapid and uncontrolled cell division, along with the formation of new blood vessels, can lead to the development of tumors.

Mutations in the *SDHA* gene were identified in a small number of people with Leigh syndrome, a progressive brain disorder that typically appears in infancy or early childhood. Affected children may experience vomiting, seizures, delayed development, muscle weakness, and problems with movement. Heart disease, kidney problems, and difficulty breathing can also occur in people with this disorder. The one child died suddenly at the age of five months from a severe deterioration of neuromuscular, cardiac, and hepatic symptoms after an intermittent infection. The *SDHA* gene mutations responsible for Leigh syndrome change single amino acids in the SDHA protein or result in an abnormally short protein. These genetic changes disrupt the activity of the SDH enzyme, impairing the ability of mitochondria to produce energy. Further studies showed that several patients with complex II deficiency do not have mutations of *SDHA*. This suggested a role of additional nuclear genes involved in synthesis, assembly, or maintenance of SDH.  It is not known, however, how mutations in the *SDHA* gene are related to the specific features of Leigh syndrome [41, 42].

Two plausible hypotheses have been proposed to explain the peculiar linkage between disruption of electron flow through mitochondrial complex II and tumorigenesis in neuroendocrine cells. In the reactive oxygen species (ROS) hypothesis, it is proposed that an intact, catalytically active SDHA subunit generates genotoxic ROS by uncoupled electron flow from succinate to oxygen or water in cells where one of the electron-carrying subunits (SDHB, SDHC or SDHD) is missing or inactive. The ROS model implies that genotoxic ROS mutagenize nuclear proto-oncogenes or tumor suppressors (Figure 11). This model predicts that ROS should be increased in cells lacking SDHB, SDHC or SDHD, but not when SDHA is missing. Although certain mutations in these genes result in ROS production in *Saccharomyces cerevisiae* and mammalian cell lines, it is not clear that ROS accumulate to levels that are mutagenic.
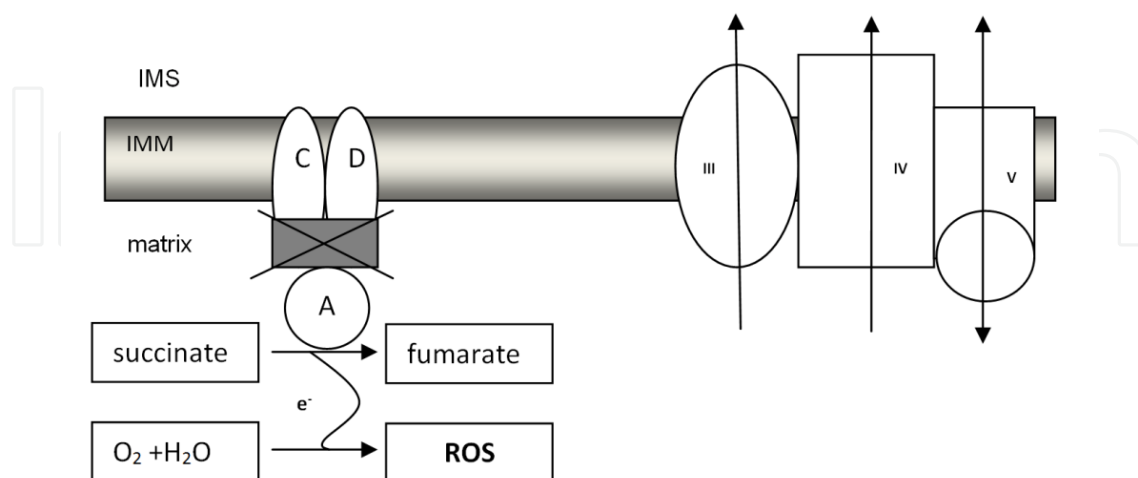


**Figure 11.** ROS model [18].

In the succinate accumulation hypothesis, the loss of SDHB results in loss of SDH activity and causes succinate accumulation (Figure 12).
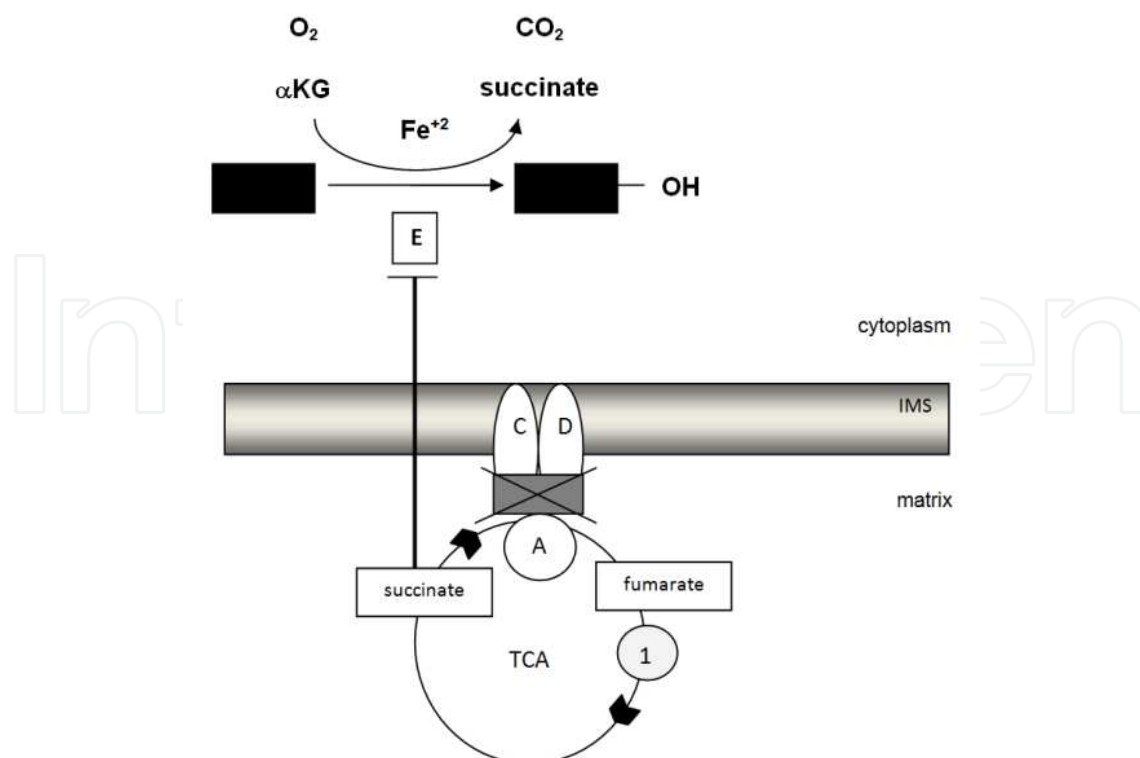
**Figure 12.** Succinate accumulation model [18].

Excess succinate is shuttled from the mitochondrial matrix to the cytoplasm, where it inhibits any of several aKG-dependent enzymes (E) that regulate levels or activities of important regulatory proteins (black box). The loss or inactivation of SDHB, C or D proteins yields a catalytically inactive SDHA subunit, resulting in blockade of the TCA cycle and diffusion of accumulated succinate to the cytoplasm. Succinate can then act as an inhibitor of a-ketoglutarate-dependent enzymes that use ferrous iron and molecular oxygen as cofactors to hydroxylate their substrates and generate succinate as a product. It has been demonstrated that two a-ketoglutarate -dependent enzymes, the prolyl hydroxylases, are inhibited by succinate accumulation in cells that have lost SDHD function. Smith et al [18] reported that yeast cells disrupted in *SDH2* (*sdh2Δ*) show increased in ROS production and protein oxidation without detectable increase in DNA damage. More strikingly, *sdh2Δ* cells dramatically accumulate succinate resulting in inhibition of at least two aKG-dependent enzymes that generate succinate as a by-product [18].

## 6. Metabolic engineering of yeast SDH for the biotechnological processes

Metabolic engineering, i.e., the intentional redirection of metabolic fluxes, plays an exceptional role in improving yeast strains for all industrial applications. In contrast to classical methods of genetic strain improvement such as selection, mutagenesis, mating, and hybridization, metabolic engineering confers two major advantages: (1) the directed modification of strains without the accumulation of unfavorable mutations and (2) the introduction of genes from foreign organisms to equip *S. cerevisiae* with novel traits. The latter is particularly crucial for industrial biotechnology to provide pathways that extend the

spectrum of usable industrial media (e.g., lignocellulosic biomass) and/or to produce compounds not naturally formed by *S. cerevisiae*. Since the first introduction of metabolic engineering, there have been tremendous enhancements of its toolbox, and several related disciplines have emerged, such as inverse metabolic engineering and evolutionary engineering. These developments have strongly influenced yeast strain improvement programs in the past few years and have greatly enhanced the potential for using yeast in biotechnological production processes [43].

The main goals of metabolic engineering can be summarized in the following four categories: (1) improvement of yield, productivity and overall cellular physiology, (2) extension of the substrate range, (3) deletion or reduction of by-product formation and (4) introduction of pathways leading to new products. Commonly these goals can be achieved by a three-step procedure. Firstly, a genetic modification is proposed, based on metabolic models. After genetic modification, the recombinant strain is analysed and the results are then used to identify the next target for genetic manipulation, if necessary. Thus, the construction of an optimal strain involves a close interaction between synthesis and analysis, usually for several consecutive rounds. The rapid development and frequent success in this field is demonstrated by the large number of reviews about the theoretical and practical aspects of metabolic engineering. Knowledge of cellular and microbial physiology, as well as the underlying metabolic networks or enzymes, is an important prerequisite for successful engineering. A new term, 'inverse metabolic engineering' (IME) coins to encompass the construction of strains with a particularly desirable physiological phenotype, e.g. enhanced production of heterologous protein [44].

Recently, a computational approach for the identification of every possible biochemical reaction from a given set of enzyme reaction rules was reported. This analysis suggested that the native pathways are thermodynamically more favorable than the alternative possible pathways. The pathways generated involve compounds that exist in biological databases, as well as compounds that exist in chemical databases and novel compounds, suggesting novel biochemical routes for these compounds and the existence of biochemical compounds that remain to be discovered or synthesized through enzyme and pathway engineering [45].

Due to its importance in traditional biotechnology such as baking, brewing, and wine making, research activities historically have focused on the yeast *Saccharomyces cerevisiae*. It is relatively tolerant to low pH values and high sugar and ethanol concentrations, i.e., properties which lower the risk of contamination in industrial fermentation. These features are the major reasons for increasing *S. cerevisiae* exploration in industrial ("white") biotechnology, focusing on the fermentative production of industrially relevant biochemicals, e.g., glycerol, propanediol, sugar alcohols, organic acids, etc. Among these compounds, several organic acids may fulfil a role as platform molecules using their (multiple) functional groups as a target for enzymic or chemical catalysis [43].

In the United States were identified 10 organic acids as key chemical building blocks [44]. Similarly, the European focus group BREW identified 21 key compounds that can be
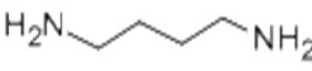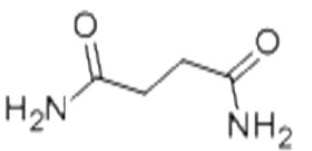
produced from different, including renewable sources, a number of which were organic acids [45]. One example of such a chemical is succinic acid. Succinic acid is used as a surfactant, detergent or foaming agent, as an ion chelator, and also in the food industry as an acidulant, flavoring agent or anti-microbial agent, as well as in health-related products (such pharmaceuticals and antibiotics). Currently, it is produced from petrol and is too expensive to be used as a general building-block chemical. However, provided that its price becomes competitive, succinic acid could replace petrol-derived maleic anhydride in chemical synthesis processes in the future [46-47]. Similar chemical derivatizations can be applied to malic and fumaric acid, so that they can also be considered interesting $C_4$ building blocks [48-53].

The chemical behavior of the dicarboxylic acid – succinic acid is determined principally by its two carboxyl groups. This substance is either directly utilized in the pharmaceutical or chemical industry or represent building block or precursor for further chemical or enzymatic syntheses. The following reactions and derivatives are considered interesting: (1) reductions of succinic acid to 1,4-butanediol, γ-butyrolactone, tetrahydrofuran and its derivatives; (2) reductive amination of succinic acid or γ-butyrolactone to pyrrolidiones; (3) polymerization of succinic acid with diols (building block of polyesters); (4) polymerisation of succinic acid with diamines to form polyamides, etc. The examples of the substances that can be derived from succinic acid are shown in table 2.

Butanediol, tetrahydrofuran and γ-butyrolactone, are standard substances for the chemical industry. These are used as solvents, as well as for fiber and polymer production. Dimethylsuccinate is one of the so-called dibasic esters that have great potential as solvents with environmentally benign characteristics. Thus, the potential market volume for succinic acid is high, fuelling substantial efforts to establish a microbial process for succinic acid production [47].

The chemical synthesis of succinic acid is predominantly based on maleic anhydride and requires heavy metal catalysts, organic solvents, high temperatures and high pressures. It makes the conversion of maleic anhydride to succinic acid costly and ecologically questionable [49]. On the other hand, succinate is produced naturally by many microorganisms as an intermediate of the central metabolism or as a fermentation end product.

The succinate producers include bacterial strains, e.g. *Mannheimia succiniproducens* [54]. However, none of these microorganisms are currently used in industry. Some prokaryotes

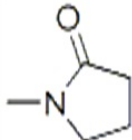| No | Name | Chemical structure | Examples of uses |
|----|------|--------------------|------------------|
| 1 | 1,4 -Diaminobutane | $H_2N$ ⌒⌒ $NH_2$ | Used for polyamide production |
| 2 | Succindiamide | $H_2N$—C(=O)—CH$_2$—CH$_2$—C(=O)—$NH_2$ | Used as anticonvulsant drugs and to form covalent bonds between proteins or peptides and plastics |

| No | Name | Chemical structure | Examples of uses |
|---|---|---|---|
| 3 | 1,4 – Butanediol (BDO) | | Replacement of fossil-derived BDO by bio-based BDO in polybutylene tereftalate (PBT) Replacement of fossil-derived BDO by bio-based BDO in polybutylene succinate (PBS) |
| 4 | Succinonitrile | | Precurser for industrial production of polyamides |
| 5 | Dimethylsuccinate | | Used as fuel oxygenates and as green solvents |
| 6 | N-methyl-pyrolidone (NMP) | | Replacement of fossil-derived NMP by bio-based NMP; NMP is an important, versatile solvent for the chemical industry |
| 8 | 2-Pyrolidone | | As above |
| 7 | γ-Butyrolactone (GBL) | | Replacement of fossil-derived BGL by bio-based BGL ; BGL is important as an intermediate in the manufacture of pyrrolidone derivatives and as a solvent for polymers and agrochemicals |
| 9 | Tetrahydrofuran (THF) | | Replacement of fossil-derived THF by bio-based THF ; THF is mainly used as a solvent and as an intermediate in the production of thermoplastic polyurethanes, elastic fibers etc. |
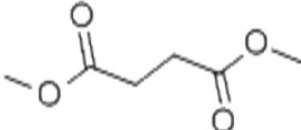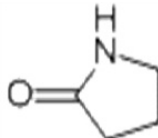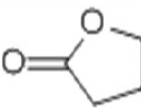
**Table 2.** Examples of various substances that can be derived from succinic acid by chemical conversion [47].

have complex medium requirements and they are generally unable to grow and produce organic acids at the low pH values. These restrictions provide strong incentives to integrate and optimize succinate production pathways in other microorganisms via metabolic engineering approaches.

The popularity of *S. cerevisiae* in basic and applied research is undoubtedly influenced by its classification as GRAS (generally regarded as safe) by the U.S. Food and Drug Administration (FDA). Baker's yeast *S. cerevisiae* was the first eukaryotic organism in which complete genomic sequence was determined. Several databases such as the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) and the Comprehensive Yeast Genome Database (http://mips.gsf.de/genre/proj/yeast/) contain an enormous amount of information concerning *S. cerevisiae* genes, open reading frames, and gene products. The yeast *S. cerevisiae* became a well established eukaryotic model organism to study fundamental biological processes such as aging, mRNA transport, the cell cycle, and many more. *Saccharomyces cerevisiae* grows well in a simple chemically defined medium, under acidic conditions, even at pH values equal 3. At such low pH values, many weak acids, including succinate, occur predominantly in their undissociated form. This is advantageous for industrial production, as it reduces the need for titration with alkali and allows for direct recovery of undissociated acids. Consequently, there is no need for large quantities of acidifying agents, and the formation of salt byproducts (e.g. gypsum) is strongly reduced. In addition, *S. cerevisiae* robust tolerance in acidic conditions represents a major advantage in that it lowers the risk of contamination in industrial fermentation [44, 49].

The yeast-based fermentation process, which operates at a much lower pH than competing processes, allows succinic acid to be produced with a significantly higher energy efficiency compared to the traditional method. This compound is not accumulated intracellularly. It is also one of the first bio-based processes that sequesters carbon dioxide in the production process [47, 53]. This makes the yeast *Saccharomyces cerevisiae* a suitable and promising candidate for the biotechnological production of succinic acid on an industrial scale.

The metabolic engineering strategy was used for the oxidative production of succinic acid by deletion SDH1, SDH2 genes in the genome. Arikawa et al. [55] reported an increased succinic acid productivity in sake yeast strains with deletions of TCA cycle genes. In comparison to the wild-type, succinate levels were increased up to2.7 fold in a strain with simultaneous disruption of a subunit of succinate dehydrogenase (SDH1) and fumarase (FUM1) under aerobic conditions. The single deletion of gene SDH1 led to a1.6-fold increase of succinic acid production. In another study on sake yeast strains, the deletion of genes encoding for succinate dehydrogenase subunits (SDH1, SDH2, SDH3, and SDH4) also resulted inincreased succinate productivity in aerobic conditions. Raab et al. [48] reported the construction of yeast strains for the biotechnological production of succinic acid. The genes *SDH1, SDH2, IDH1* and *IDP1*, which encode mitochondrial enzymes were deleted with the aim to disrupt succinate and isocitrate dehydrogenase activity to redirect the carbon flux and to allow succinate to accumulate as an end-product. This study showed that the yeast *S. cerevisiae* is capable of synthesizing significant amounts of succinic acid, which is exported quantitatively into the culture broth and not being accumulated intracellularly.

The constructed yeast strains with disruptions in the TCA cycle produced succinic acid up to 3.62 g/L at a yield of 0.11 mol /mol glucose in shake flask cultures.

*Saccharomyces cerevisiae* is one of the most highly researched model organisms in different biological studies. Using this yeast we can effectively re-examine long-standing and fundamental questions regarding regulation of metabolism and prediction of dynamic models in various cells, including mammalian tissues. This is a considerable knowledge about the composition, enzymology and membrane binding of the enzyme and relatively new discoveries about its genetics and biosynthesis. Through such efforts, we are able to identify key features of cellular metabolic pathways which can be use both in medicine and in different biotechnological processes.

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