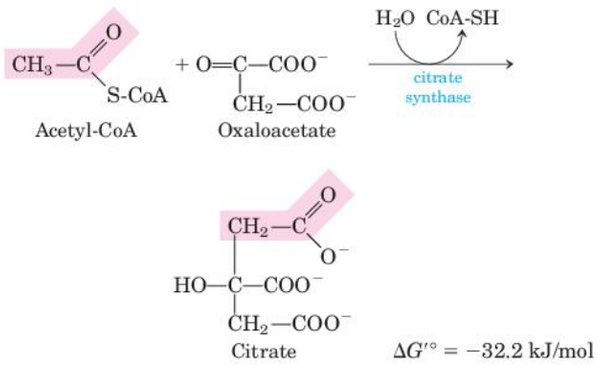
oxidative reactions and of the coupled synthesis of ATP. In photosynthetic eukaryotes, mitochondria are the major site of ATP production in the dark, but in daylight chloroplasts produce most of the organism's ATP. In most prokaryotes, the enzymes of the citric acid cycle are in the cytosol, and the plasma membrane plays a role analogous to that of the inner mitochondrial membrane in ATP synthesis (Chapter 19).

The Citric Acid Cycle Has Eight Steps

In examining the eight successive reaction steps of the citric acid cycle, we place special emphasis on the chemical transformations taking place as citrate formed from acetyl-CoA and oxaloacetate is oxidized to yield CO_2 and the energy of this oxidation is conserved in the reduced coenzymes NADH and $FADH_2$.

① *Formation of Citrate* The first reaction of the cycle is the condensation of acetyl-CoA with **oxaloacetate** to form **citrate,** catalyzed by **citrate synthase:**



In this reaction the methyl carbon of the acetyl group is joined to the carbonyl group (C-2) of oxaloacetate. Citroyl-CoA is a transient intermediate formed on the active site of the enzyme (see Fig. 16–9). It rapidly undergoes hydrolysis to free CoA and citrate, which are released from the active site. The hydrolysis of this high-energy thioester intermediate makes the forward reaction highly exergonic. The large, negative standard free-energy change of the citrate synthase reaction is essential to the operation of the cycle because, as noted earlier, the concentration of oxaloacetate is normally very low. The CoA liberated in this reaction is recycled to participate in the oxidative decarboxylation of another molecule of pyruvate by the PDH complex.

Citrate synthase from mitochondria has been crystallized and visualized by x-ray diffraction in the presence and absence of its substrates and inhibitors (Fig. 16–8). Each subunit of the homodimeric enzyme is a single polypeptide with two domains, one large and rigid, the other smaller and more flexible, with the active site between them. Oxaloacetate, the first substrate to bind to the enzyme, induces a large conformational

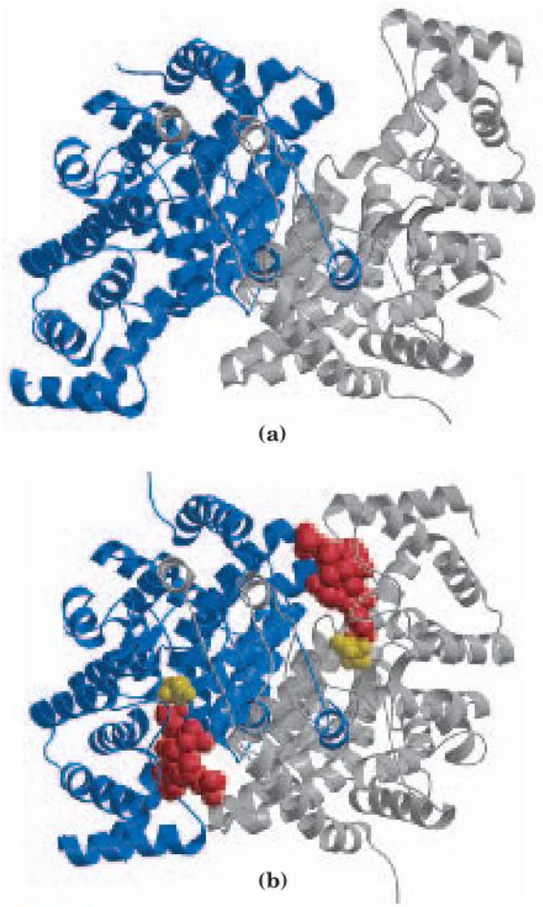
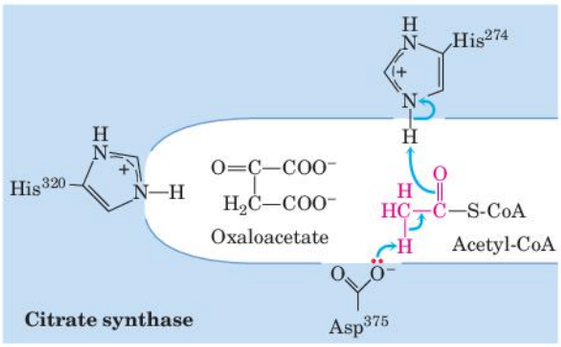


FIGURE 16–8 Structure of citrate synthase. The flexible domain of each subunit undergoes a large conformational change on binding oxaloacetate creating a binding site for acetyl-CoA. **(a)** open form of the enzyme alone (PDB ID 5CSC); **(b)** closed form with bound oxaloacetate (yellow) and a stable analog of acetyl-CoA (carboxymethyl-CoA; red) (derived from PDB ID 5CTS).

change in the flexible domain, creating a binding site for the second substrate, acetyl-CoA. When citroyl-CoA has formed in the enzyme active site, another conformational change brings about thioester hydrolysis, releasing CoA-SH. This induced fit of the enzyme first to its substrate and then to its reaction intermediate decreases the likelihood of premature and unproductive cleavage of the thioester bond of acetyl-CoA. Kinetic studies of the enzyme are consistent with this ordered bisubstrate mechanism (see Fig. 6–13). The reaction catalyzed by citrate synthase is essentially a Claisen condensation (p. 485), involving a thioester (acetyl-CoA) and a ketone (oxaloacetate) (Fig. 16–9).

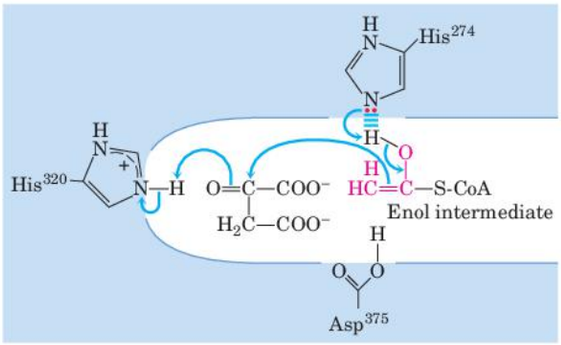
② *Formation of Isocitrate via cis-Aconitate* The enzyme **aconitase** (more formally, **aconitate hydratase**) catalyzes the reversible transformation of citrate to **isocitrate,** through the intermediary formation of the tricarboxylic acid ***cis*-aconitate,** which normally does



The thioester linkage in acetyl-CoA activates the methyl hydrogens, and Asp^{375} abstracts a proton from the methyl group, forming an enolate intermediate.

①

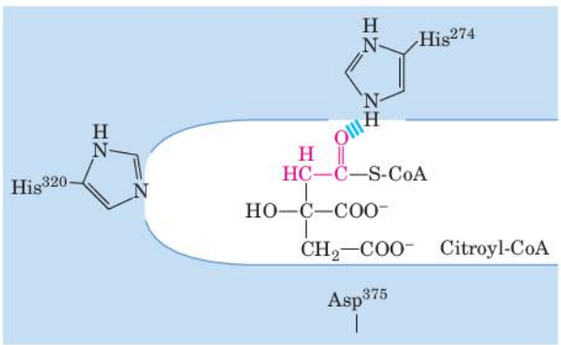
The intermediate is stabilized by hydrogen bonding to and/or protonation by His^{274} (full protonation is shown).

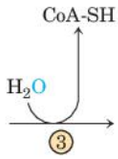


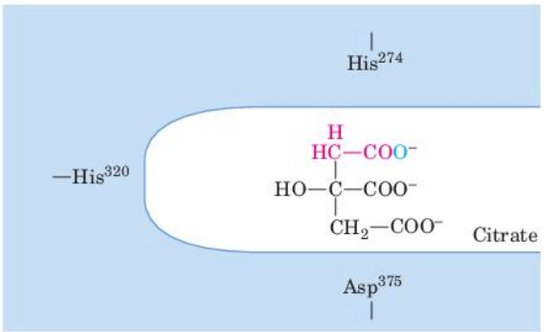
The enol(ate) rearranges to attack the carbonyl carbon of oxaloacetate, with His^{274} positioned to abstract the proton it had previously donated. His^{320} acts as a general acid.

②

The resulting condensation generates citroyl-CoA.

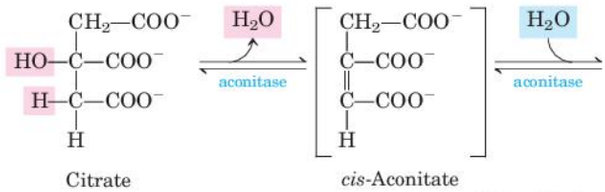


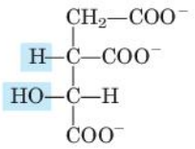




The thioester is subsequently hydrolyzed, regenerating CoA-SH and producing citrate.

not dissociate from the active site. Aconitase can promote the reversible addition of H_2O to the double bond of enzyme-bound *cis*-aconitate in two different ways, one leading to citrate and the other to isocitrate:





$\Delta G'^{\circ} = 13.3$ kJ/mol

Although the equilibrium mixture at pH 7.4 and 25 °C contains less than 10% isocitrate, in the cell the reaction is pulled to the right because isocitrate is rapidly consumed in the next step of the cycle, lowering its steady-state concentration. Aconitase contains an **iron-sulfur center** (Fig. 16–10), which acts both in the binding of the substrate at the active site and in the catalytic addition or removal of H_2O.

MECHANISM FIGURE 16–9 **Citrate synthase.** In the mammalian citrate synthase reaction, oxaloacetate binds first, in a strictly ordered reaction sequence. This binding triggers a conformation change that opens up the binding site for acetyl-CoA. Oxaloacetetate is specifically oriented in the active site of citrate synthase by interaction of its two carboxylates with two positively charged Arg residues (not shown here). The details of the mechanism are described in the figure. Citrate Synthase Mechanism

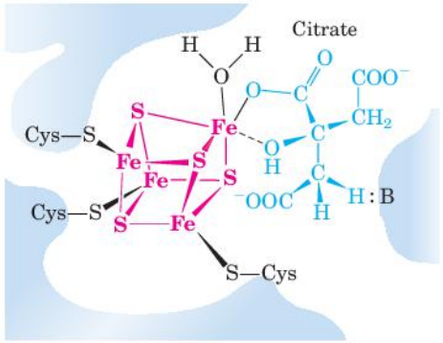
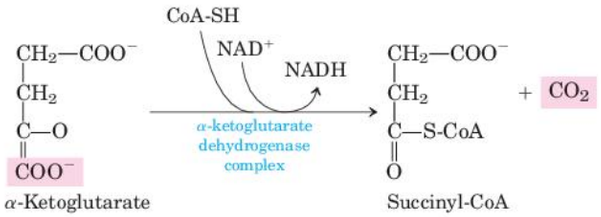


FIGURE 16–10 Iron-sulfur center in aconitase. The iron-sulfur center is in red, the citrate molecule in blue. Three Cys residues of the enzyme bind three iron atoms; the fourth iron is bound to one of the carboxyl groups of citrate and also interacts noncovalently with a hydroxyl group of citrate (dashed bond). A basic residue (:B) on the enzyme helps to position the citrate in the active site. The iron-sulfur center acts in both substrate binding and catalysis. The general properties of iron-sulfur proteins are discussed in Chapter 19 (see Fig. 19–5).

③ Oxidation of Isocitrate to α-Ketoglutarate and CO_2 In the next step, **isocitrate dehydrogenase** catalyzes oxidative decarboxylation of isocitrate to form ***α*-ketoglutarate** (Fig. 16–11). Mn^{2+} in the active site interacts with the carbonyl group of the intermediate oxalosuccinate, which is formed transiently but does not leave the binding site until decarboxylation converts it to α-ketoglutarate. Mn^{2+} also stabilizes the enol formed transiently by decarboxylation.

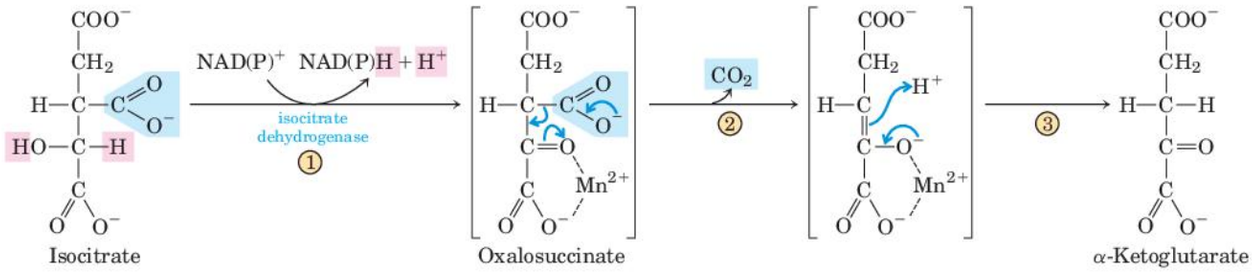
There are two different forms of isocitrate dehydrogenase in all cells, one requiring NAD^+ as electron acceptor and the other requiring $NADP^+$. The overall reactions are otherwise identical. In eukaryotic cells, the NAD-dependent enzyme occurs in the mitochondrial matrix and serves in the citric acid cycle. The main function of the NADP-dependent enzyme, found in both the mitochondrial matrix and the cytosol, may be the generation of NADPH, which is essential for reductive anabolic reactions.

④ Oxidation of α-Ketoglutarate to Succinyl-CoA and CO_2 The next step is another oxidative decarboxylation, in which α-ketoglutarate is converted to **succinyl-CoA** and CO_2 by the action of the ***α*-ketoglutarate dehydrogenase complex;** NAD^+ serves as electron acceptor and CoA as the carrier of the succinyl group. The energy of oxidation of α-ketoglutarate is conserved in the formation of the thioester bond of succinyl-CoA:



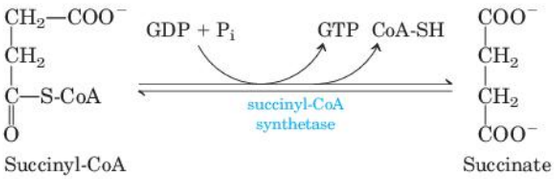
$\Delta G'^\circ = -33.5$ kJ/mol

This reaction is virtually identical to the pyruvate dehydrogenase reaction discussed above, and the α-ketoglutarate dehydrogenase complex closely resembles the PDH complex in both structure and function. It includes three enzymes, homologous to E_1, E_2, and E_3 of the PDH complex, as well as enzyme-bound TPP, bound lipoate, FAD, NAD, and coenzyme A. Both complexes are certainly derived from a common evolutionary ancestor. Although the E_1 components of the two complexes are structurally similar, their amino acid sequences differ and, of course, they have different binding specificities: E_1 of the PDH complex binds pyruvate, and E_1 of the α-ketoglutarate dehydrogenase complex binds α-ketoglutarate. The E_2 components of the two complexes are also very similar, both having covalently bound lipoyl moieties. The subunits of E_3 are identical in the two enzyme complexes.



MECHANISM FIGURE 16–11 Isocitrate dehydrogenase. In this reaction, the substrate, isocitrate, loses one carbon by oxidative decarboxylation. In step ①, isocitrate binds to the enzyme and is oxidized by hydride transfer to NAD^+ or $NADP^+$, depending on the isocitrate dehydrogenase isozyme. (See Fig. 14–12 for more information on hydride transfer reactions involving NAD^+ and $NADP^+$.) The resulting carbonyl group sets up the molecule for decarboxylation in step ②. Interaction of the carbonyl oxygen with a bound Mn^{2+} ion increases the electron-withdrawing capacity of the carbonyl group and facilitates the decarboxylation step. The reaction is completed in step ③ by rearrangement of the enol intermediate to generate α-ketoglutarate.

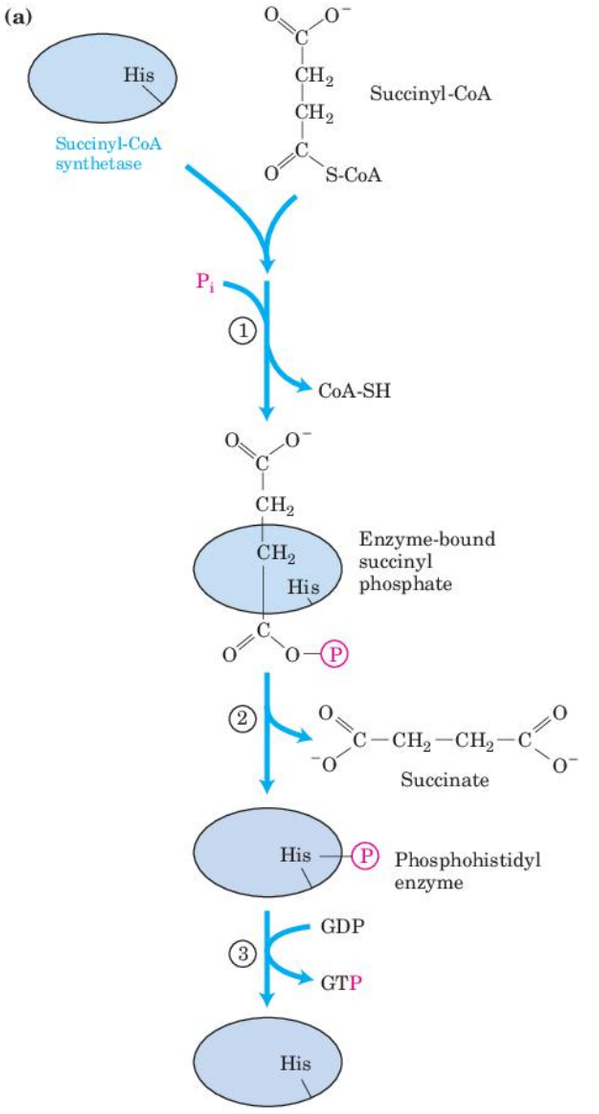
⑤ *Conversion of Succinyl-CoA to Succinate* Succinyl-CoA, like acetyl-CoA, has a thioester bond with a strongly negative standard free energy of hydrolysis ($\Delta G'^{\circ} \approx -36$ kJ/mol). In the next step of the citric acid cycle, energy released in the breakage of this bond is used to drive the synthesis of a phosphoanhydride bond in either GTP or ATP, with a net $\Delta G'^{\circ}$ of only −2.9 kJ/mol. **Succinate** is formed in the process:



$$\Delta G'^{\circ} = -2.9 \text{ kJ/mol}$$

The enzyme that catalyzes this reversible reaction is called **succinyl-CoA synthetase** or **succinic thiokinase;** both names indicate the participation of a nucleoside triphosphate in the reaction (Box 16–1).

This energy-conserving reaction involves an intermediate step in which the enzyme molecule itself becomes phosphorylated at a His residue in the active site (Fig. 16–12a). This phosphoryl group, which has a high group transfer potential, is transferred to ADP (or GDP) to form ATP (or GTP). Animal cells have two isozymes of succinyl-CoA synthetase, one specific for ADP and the other for GDP. The enzyme has two subunits, α (M_r 32,000), which has the Ⓟ-His residue (His^{246}) and the binding site for CoA, and β (M_r 42,000), which confers specificity for either ADP or GDP. The active site is at the interface between subunits. The crystal structure of succinyl-CoA synthetase reveals two "power helices" (one from each subunit), oriented so that their electric dipoles situate partial positive charges close to the negatively charged Ⓟ-His (Fig. 16–12b), stabilizing the phosphoenzyme intermediate. (Recall the similar role of helix dipoles in stabilizing K^+ ions in the K^+ channel (see Fig. 11–48).)



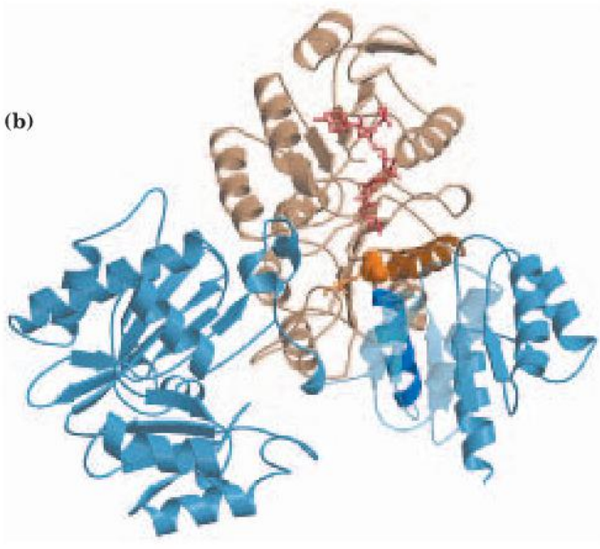


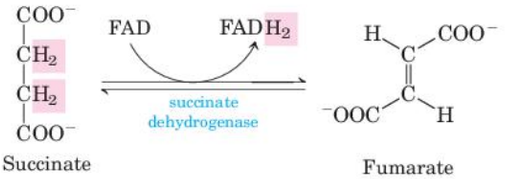
FIGURE 16–12 The succinyl-CoA synthetase reaction. (a) In step ① a phosphoryl group replaces the CoA of succinyl-CoA bound to the enzyme, forming a high-energy acyl phosphate. In step ② the succinyl phosphate donates its phosphoryl group to a His residue on the enzyme, forming a high-energy phosphohistidyl enzyme. In step ③ the phosphoryl group is transferred from the His residue to the terminal phosphate of GDP (or ADP), forming GTP (or ATP). **(b)** Succinyl-CoA synthetase of *E. coli* (derived from PDB ID 1SCU). The bacterial and mammalian enzymes have similar amino acid sequences and presumably have very similar three-dimensional structures. The active site includes part of both the α (blue) and β (brown) subunits. The power helices (bright blue, dark brown) situate the partial positive charges of the helix dipole near the phosphate group (orange) on His^{246} of the α chain, stabilizing the phosphohistidyl enzyme. Coenzyme A is shown here as a red stick structure. (To improve the visibility of the power helices, some nearby secondary structures have been made transparent.)

The formation of ATP (or GTP) at the expense of the energy released by the oxidative decarboxylation of α-ketoglutarate is a substrate-level phosphorylation, like the synthesis of ATP in the glycolytic reactions catalyzed by glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase (see Fig. 14–2). The GTP formed by succinyl-CoA synthetase can donate its terminal phosphoryl group to ADP to form ATP, in a reversible reaction catalyzed by **nucleoside diphosphate kinase** (p. 505):

$$GTP + ADP \longrightarrow GDP + ATP \qquad \Delta G'^{\circ} = 0 \text{ kJ/mol}$$

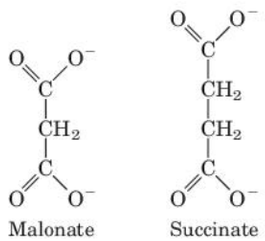
Thus the net result of the activity of either isozyme of succinyl-CoA synthetase is the conservation of energy as ATP. There is no change in free energy for the nucleoside diphosphate kinase reaction; ATP and GTP are energetically equivalent.

⑥ Oxidation of Succinate to Fumarate The succinate formed from succinyl-CoA is oxidized to **fumarate** by the flavoprotein **succinate dehydrogenase:**

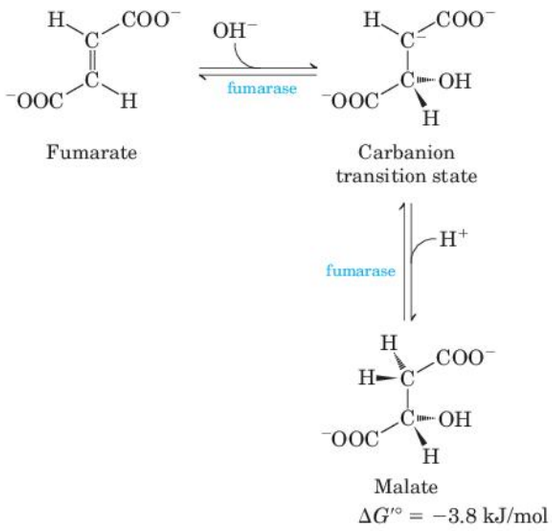


$\Delta G'^{\circ} = 0$ kJ/mol

In eukaryotes, succinate dehydrogenase is tightly bound to the inner mitochondrial membrane; in prokaryotes, to the plasma membrane. The enzyme contains three different iron-sulfur clusters and one molecule of covalently bound FAD (see Fig. 19–xx). Electrons pass from succinate through the FAD and iron-sulfur centers before entering the chain of electron carriers in the mitochondrial inner membrane (or the plasma membrane in bacteria). Electron flow from succinate through these carriers to the final electron acceptor, O_2, is coupled to the synthesis of about 1.5 ATP molecules per pair of electrons (respiration-linked phosphorylation). Malonate, an analog of succinate not normally present in cells, is a strong competitive inhibitor of succinate dehydrogenase and its addition to mitochondria blocks the activity of the citric acid cycle.

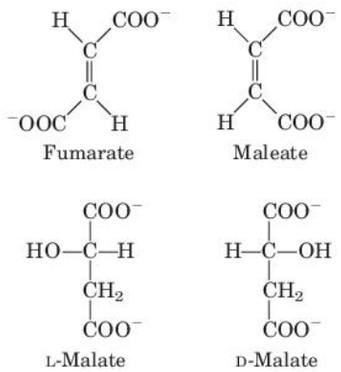


⑦ Hydration of Fumarate to Malate The reversible hydration of fumarate to **L-malate** is catalyzed by **fumarase** (formally, **fumarate hydratase**). The transition state in this reaction is a carbanion:

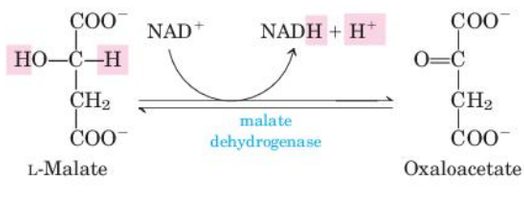


$\Delta G'^{\circ} = -3.8$ kJ/mol

This enzyme is highly stereospecific; it catalyzes hydration of the trans double bond of fumarate but not the cis double bond of maleate (the cis isomer of fumarate). In the reverse direction (from L-malate to fumarate), fumarase is equally stereospecific: D-malate is not a substrate.



⑧ Oxidation of Malate to Oxaloacetate In the last reaction of the citric acid cycle, NAD-linked **L-malate dehydrogenase** catalyzes the oxidation of L-malate to oxaloacetate:



$\Delta G'^{\circ} = 29.7$ kJ/mol

The equilibrium of this reaction lies far to the left under standard thermodynamic conditions, but in intact cells