

macroautophagy via a mitogen-activated protein (MAP) kinase-mediated mechanism. The other mechanism involves amino acids (e.g. leucine, phenylalanine and tyrosine) that are not concentrated within the liver but appear to exert their actions via an mTOR-mediated pathway.

Recent studies have clarified the mechanism by which glutamine and system A substrates affect macroautophagy. The accumulation of these amino acids within cells, together with the cotransported Na<sup>+</sup> (which is partly exchanged for K<sup>+</sup>), is followed by a flux of water into the cell to equilibrate the osmotic pressure on both sides of the cell membrane. The resulting increase in cell volume (cell hydration) results in an immediate activation of MAP kinases, in particular ERK and p38<sup>MAPK</sup>. This latter kinase brings about the inhibition of macroautophagy at the level of the formation of the autophagosome [17]. More work is needed to elucidate the complete mechanism, but the key role of cell swelling is confirmed by experiments that demonstrated a comparable degree of inhibition of proteolysis when cells were swollen in hypo-osmotic media.

Amino acids such as leucine, phenylalanine and tyrosine are agonists for another mechanism whereby amino acids inhibit macroautophagy [16,18]. The mechanism appears to involve mTOR, as rpS6, which is downstream from mTOR, becomes rapidly phosphorylated with the same kinetics as the inhibition of proteolysis. Rapamycin, an inhibitor of mTOR, could partly overcome the inhibition of autophagic proteolysis by amino acids. Again, much work remains before we will have a complete picture of the mechanism. The phosphorylation of rpS6 does not implicate this protein in the inhibition of macroautophagy; rather, it should be taken as indicating an efficient regulatory system in which mTOR activation plays a role in both the stimulation of protein synthesis and the suppression of proteolysis. There is also evidence for an mTOR-independent mechanism whereby these amino acids can suppress macroautophagy. This appears to be another example of redundant cell signalling systems. Figure 3 summarizes these mechanisms.

These new insights into amino acid-mediated cell signalling have considerable physiological significance. Amino acids signal their availability for anabolic processes, not only by being available as substrates, but also by more sophisticated mechanisms. Simultaneously, they signal the need to suppress catabolic processes. mTOR and cell hydration emerge as important 'interpreters' of amino acid availability. Recent studies also suggest that leucine may act via mTOR-independent mechanisms. It is clear that mTOR is not only an amino acid-responsive kinase but also a more general nutrient sensor; it can be antagonized by the adenosine monophosphate (AMP)-stimulated kinase which responds to the AMP/ATP ratio. The interaction and synergy of amino acid signalling with that of insulin remains to be fully elucidated. Finally, a crucial unsolved issue is the identification of the precise molecule(s) that interact(s) with regulatory amino acids such as leucine. However, it can be confidently asserted that recent work on amino acid signalling has revealed a new vista in amino acid metabolism that is likely to become even more important in the future.

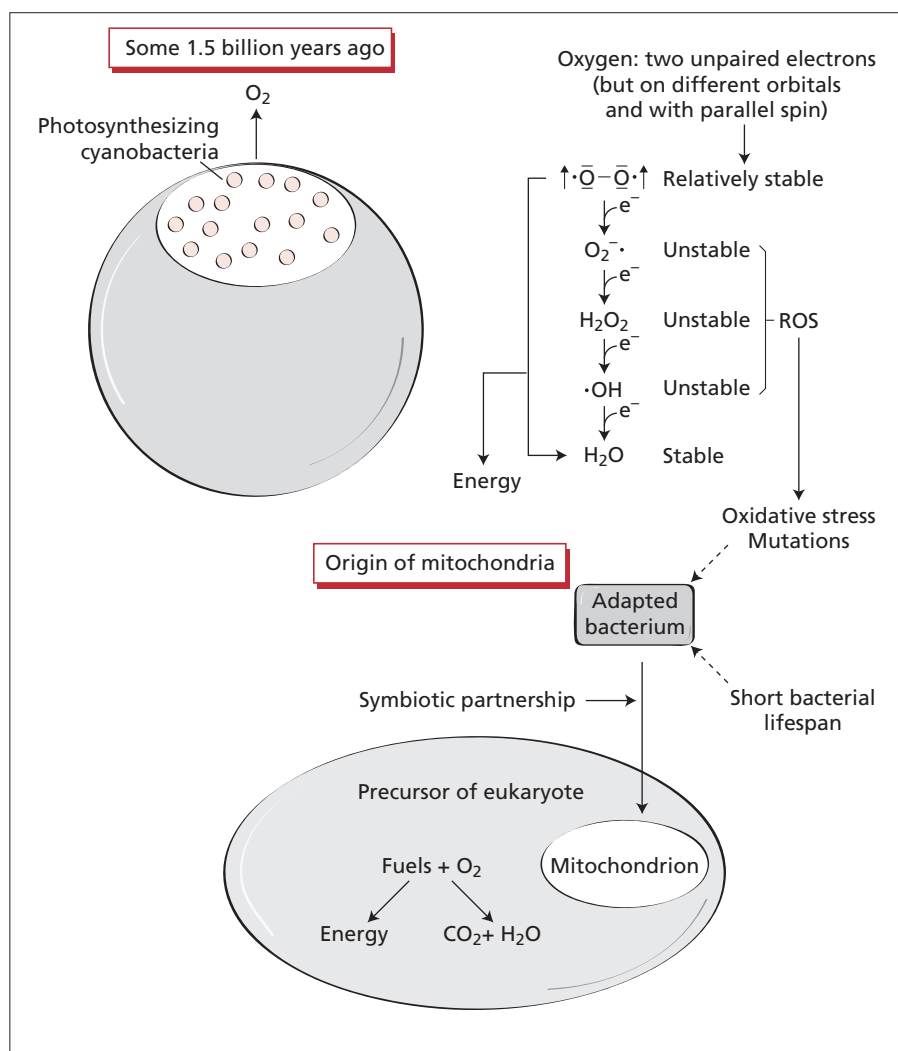
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### 2.3.4 Mitochondria and energy formation

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Mitochondria are double-membrane organelles derived evolutionarily from bacteria. They have their own DNA and reproduce asexually, but only women transmit mitochondrial DNA. Mitochondria represent remarkable biochemical machines that are able to harness the energy produced by the oxidation of fat and other energetic substrates in a form usable by the cell.



**Fig. 1** Oxygen and the origin of mitochondria. Some 1.5 billion years ago, photosynthesizing cyanobacteria began releasing oxygen in the atmosphere. Although oxygen is a diradical, it is relatively stable because its two unpaired electrons are located on different orbitals and have a parallel spin. Yet, oxygen is avid for electrons. Its full reduction into water (by four successive electrons) releases considerable energy. However, its incomplete reduction by one, two or three electrons produces reactive oxygen species (ROS), which cause oxidative stress and gene mutations. The resulting high mutation rate, combined with the short lifespan and rapid turnover of bacteria, has enabled them to adapt quickly to the oxidizing environment. Thanks to a symbiotic partnership, these adapted bacteria have become our present-day mitochondria. They indirectly burn fuels with oxygen to provide considerable energy, with limited ROS formation and no immediate toxicity.

## Origin and structure of mitochondria

Some 1.5 billion years ago, photosynthesizing cyanobacteria began releasing oxygen in the earth's atmosphere (Fig. 1) [1]. Oxygen is an atypical molecule (Fig. 1). Although it is a diradical, its two unpaired electrons are located on different orbitals and have a parallel spin, making oxygen a relatively stable molecule [2]. Yet, oxygen is avid for electrons. Its incomplete reduction by one, two or three successive electrons produces reactive oxygen species (ROS), which react with biological molecules to cause oxidative stress and gene mutations (Fig. 1) [1]. However, the full reduction of oxygen by four successive electrons forms a stable product (water) and releases considerable energy (Fig. 1). Therefore, the advent of oxygen evolutionarily was both a severe threat to life (due to the toxicity of ROS) and also a great opportunity (due to the energetic potential of fuel oxidation).

Thanks to their short lifespan and rapid turnover, bacteria took advantage of the high mutation rate triggered by ROS to adapt quickly to the new oxygen environment (Fig. 1). They

developed biochemical machines enabling them indirectly to burn fuels with oxygen and produce usable energy, while minimizing ROS formation and toxicity. Rather than trying to emulate the biochemical perfection of bacteria, other forms of life have associated with them. Through a symbiotic partnership, these adapted bacteria have become our present-day mitochondria (Fig. 1) [3].

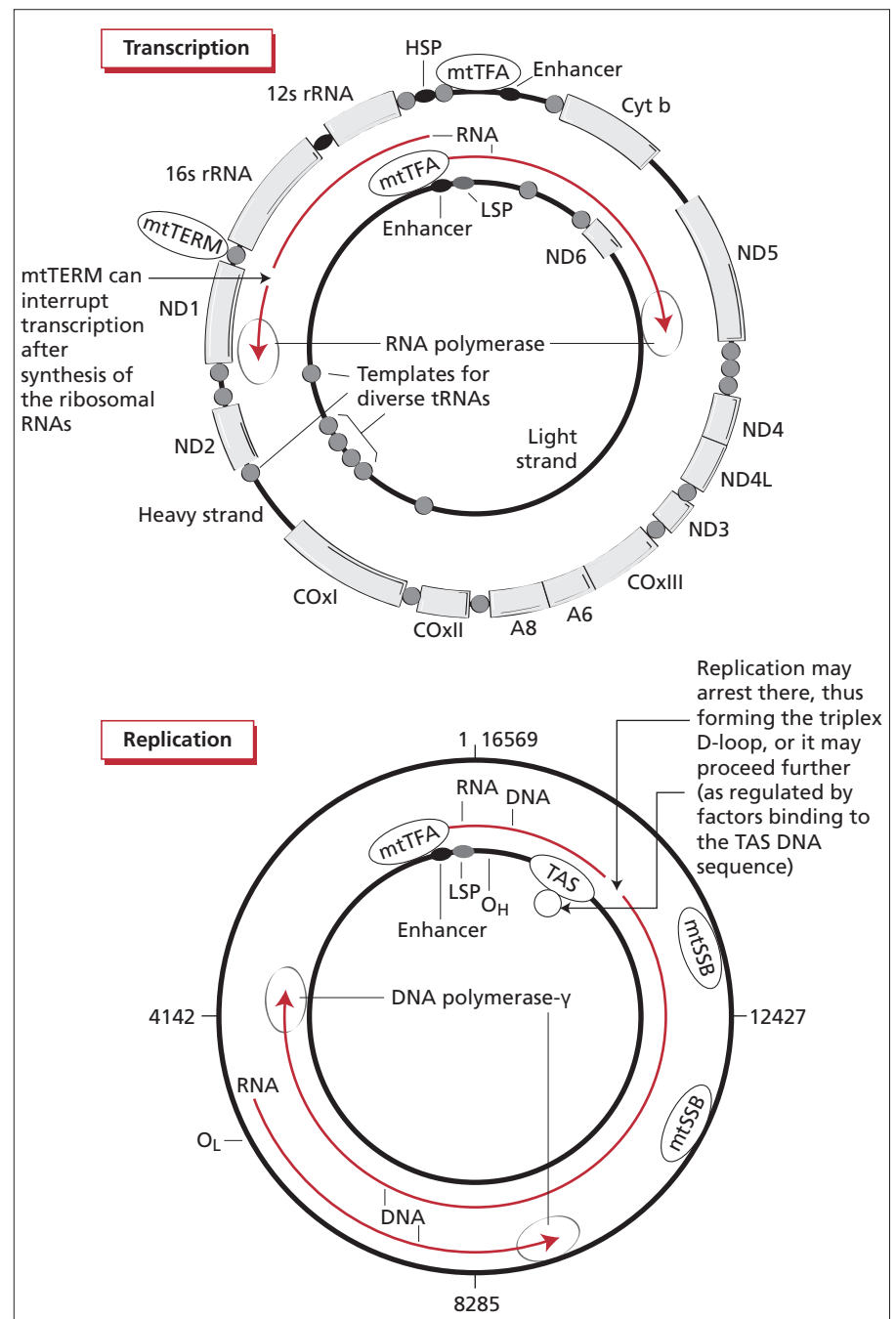
Like their bacterial ancestors, mitochondria have two membranes. The circular outer membrane surrounds the inter-membrane space, while the folded inner membrane invaginates into cristae, which protrude into the mitochondrial matrix.

Like bacteria, mitochondria also have their own circular DNA located in the mitochondrial matrix [4].

## Mitochondrial DNA (mtDNA)

Sperm cell mitochondria are ubiquitinated and are degraded in the fertilized ovum, so that mtDNA is only transmitted by women [5]. Most somatic cells, including hepatocytes, contain

**Fig. 2** Transcription and replication of the mitochondrial genome. The human mitochondrial DNA (mtDNA) is a double-stranded circular DNA of 16 569 bp. Its transcription is mediated by a mitochondrial RNA polymerase and is enhanced by the binding of mitochondrial transcription factor A (mtTFA) to enhancer elements located upstream of the heavy strand promoter (HSP) and the light strand promoter (LSP). Transcription of the heavy strand starts at nucleotide 561 (within the HSP) and proceeds counterclockwise. After synthesis of the two ribosomal RNAs, transcription may be arrested by the binding of mitochondrial termination factor (mtTERM), or it may proceed to form a large polycistronic precursor RNA encoding for 12 polypeptides and 14 tRNAs. Transcription of the light strand starts at nucleotide 407 (within the LSP) and proceeds clockwise to form a single polycistronic precursor RNA, encoding one polypeptide and eight tRNAs. The replication of mtDNA is mediated by DNA polymerase  $\gamma$  and is asymmetrical. Only the heavy strand is replicated initially. Replication starts at the origin of replication of the heavy strand ( $O_H$ ), after a short RNA primer formed by the transcriptional machinery. The need for this RNA primer explains why mtTFA also modulates the replication of mtDNA. Mitochondrial single-stranded binding protein (mtSSB) binds to the single-stranded heavy chain and also activates replication. Replication of the heavy strand proceeds clockwise. It may arrest after a few hundred basepairs (thus forming the basal 'D-loop'), probably due to the binding of regulatory factors to the termination-associated sequence (TAS). Alternatively, replication may continue to form the whole daughter heavy strand. When the displacement front has gone beyond the origin of replication of the light strand ( $O_L$ ), the replication of the light strand then starts counterclockwise after a short RNA primer introduced by a DNA primase. A6 and A8, subunit 6 or 8 of ATP synthase; cyt b, cytochrome b; COx, subunit of cytochrome c oxidase; ND, subunit of NADH dehydrogenase; rRNA, ribosomal RNA. Grey dots represent the 22 different mitochondrial tRNAs, which also serve as punctuations.



hundreds of mitochondria, and each mitochondrion usually contains a few copies of mtDNA. All mtDNA copies are identical in healthy young persons. However, in persons with inborn mitochondrial cytopathies and in elderly subjects, intact mtDNA copies coexist with mtDNA genomes with point mutations or DNA rearrangements, such as deletions or duplications.

The human mtDNA is a small (16 569 bp), circular, double-stranded DNA (Fig. 2), which is normally twisted into a supercoiled form [4]. The proportion of heavy vs. light DNA bases

differs slightly between the two mtDNA strands. Their different buoyancies on denaturing caesium chloride gradients have led to their appellation as the 'heavy strand' and the 'light strand' respectively. At the so-called 'displacement loop' (D-loop), mtDNA contains a third DNA strand. This localized triplex structure is due to the interrupted replication of the heavy strand, as discussed below.

Although most of the ancient bacterial genes have been lost, or have migrated to nuclear DNA [6], the human mtDNA still

encodes for the two ribosomal mitochondrial RNAs, all 22 mitochondrial tRNAs and 13 of the polypeptides of the respiratory chain, including adenosine triphosphate (ATP) synthase [4]. Information is tightly packed on mtDNA (Fig. 2). With the exception of a few regulatory sequences, mtDNA consists mainly of coding sequences with no introns. Furthermore, the short DNA sequences encoding for mitochondrial tRNAs also serve as punctuation dots [4].

The transcription of mtDNA is mediated by a mitochondrial RNA polymerase and is enhanced by the binding of mitochondrial transcription factor A (mtTFA) to enhancer sequences located upstream of the heavy strand promoter and the light strand promoter [4]. Mitochondrial transcription factor B (mtTFB) may favour the interaction with RNA polymerase [4]. As shown in Fig. 2, the transcription of the heavy strand proceeds counterclockwise, while the transcription of the light strand runs clockwise.

DNA polymerase  $\gamma$  replicates mtDNA. It has proofreading activity, enabling it to remove the last added nucleotide, if mispaired [4]. The replication of mtDNA is asymmetrical (Fig. 2). Only the heavy strand is initially replicated. Replication starts at the origin of replication of the heavy strand ( $O_H$ ), after a short RNA primer. This primer is formed by the transcriptional machinery and begins at the site for the initiation of transcription of the heavy strand. The need for this RNA primer explains why mtTFA enhances not only the transcription, but also the replication of mtDNA (Fig. 2). Other factors enhancing mtDNA replication are mitochondrial single-stranded binding protein (mtSSB) and the DNA helicase, twinkle. Replication of the heavy strand proceeds clockwise. It can arrest after a few hundred basepairs, thus forming the basal D-loop, or can proceed further on. The arrest or continuation of replication may be modulated by the binding of regulatory factors to the termination-associated sequence (TAS) (Fig. 2). When about two-thirds of the heavy strand has been replicated, and the displacement front has gone past the origin of replication of the light strand ( $O_L$ ), the light strand begins to be replicated counterclockwise, after a short RNA primer introduced by a mitochondrial DNA primase [4].

Whereas mitochondria contain all the enzymes necessary for base excision repair, they cannot remove some bulky DNA lesions, such as pyrimidine dimers and interstrand cross-links [7,8]. However, heavily damaged mtDNA molecules can be degraded by mitochondrial nucleases, and then replaced by the synthesis of new mtDNA molecules replicated from unaltered mtDNA templates [8].

## Mitochondrial protein import

With the exception of the 13 polypeptides encoded by mtDNA, the thousand or so other proteins present in mitochondria are encoded by nuclear DNA, synthesized within the cytoplasm and imported into the mitochondria, either cotranslationally or posttranslationally (Fig. 3) [9,10]. Although all imported polypeptides enter the mitochondria through the Tom40

(translocase of outer membrane 40) complex, import routes then differ with the destination of the protein (Fig. 3) [9].

- Polypeptides that are destined to form  $\beta$ -barrel proteins inside the outer membrane are carried by small Tim (translocase of the inner membrane) proteins of the intermembrane space to the SAM (sorting and assembly machinery) complex, which inserts these proteins into the outer membrane (Fig. 3).
- Polypeptides that are destined to form multispanning proteins in the inner membrane are carried by the same small Tim proteins to the Tim22 (translocase of inner membrane 22) complex (also called the 'twin-pore translocase' because of its two pores), which inserts these proteins inside the inner membrane (Fig. 3) [9].
- Finally, polypeptides that are destined for the mitochondrial matrix are usually synthesized with an N-terminal, positively charged presequence achieving an  $\alpha$ -helical configuration. These polypeptides cross the inner mitochondrial membrane through the Tim23 complex (also called the 'presequence translocase'). Their import is driven by the membrane potential and also by an ATP-driven pulling mechanism mediated by mitochondrial Hsp70, which is bound to Tim44. Mitochondrial processing peptidase (MPP) then removes the presequence inside the matrix (Fig. 3).

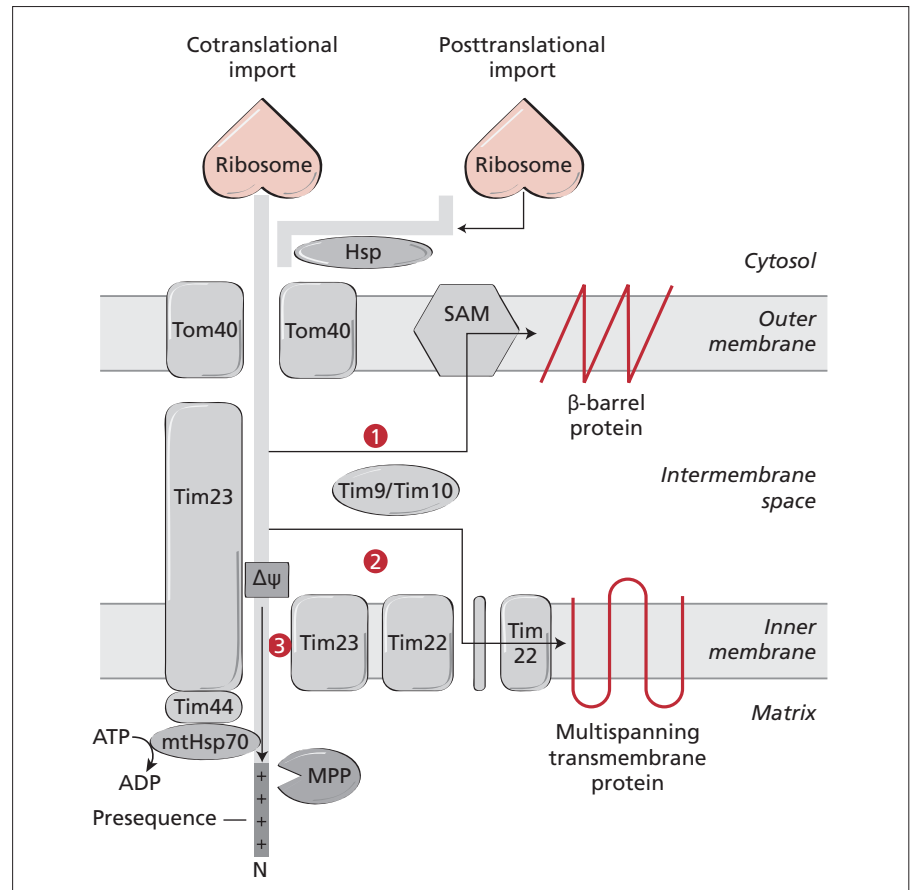
Most enzymes responsible for the tricarboxylic acid cycle, and all the proteins or enzymes involved in the replication, stability, translation and repair of mtDNA, are targeted to the mitochondrial matrix. Respiratory chain polypeptides are targeted to the inner mitochondrial membrane, except for cytochrome *c*, which is targeted to the intermembrane space but mostly associates with cardiolipin on the cytosolic face of the mitochondrial inner membrane. Finally, some  $\beta$ -oxidation enzymes are targeted to the mitochondrial inner membrane, while others are directed to the mitochondrial matrix.

## Mitochondrial $\beta$ -oxidation of fatty acids

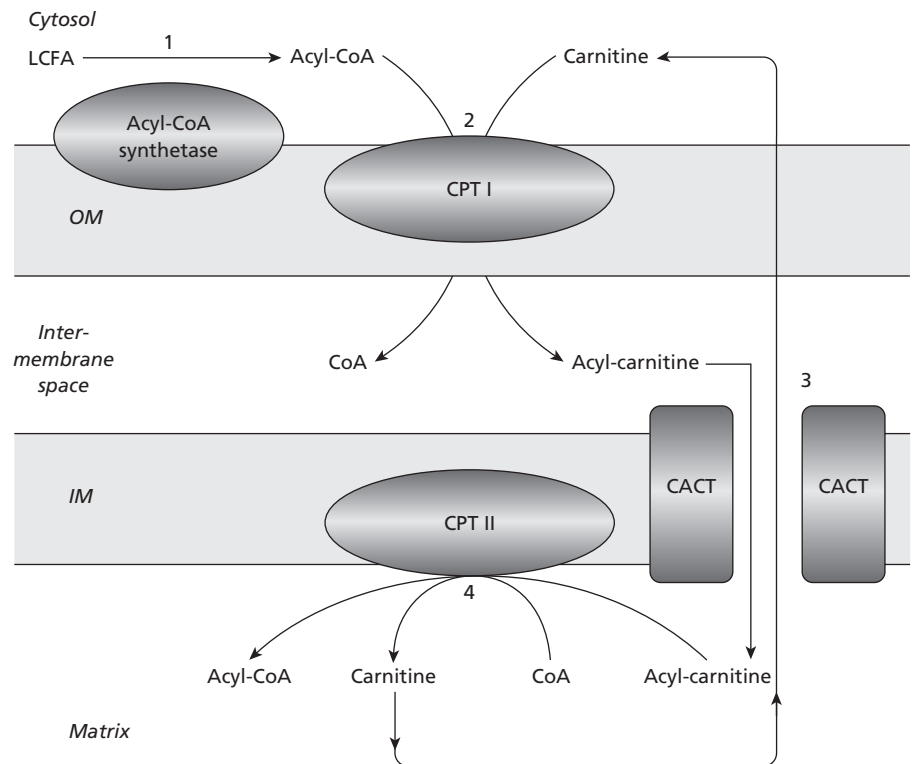
Although peroxisomes contribute to the oxidation of fatty acids (particularly that of very-long-chain fatty acids), fatty acid oxidation mainly occurs within mitochondria.

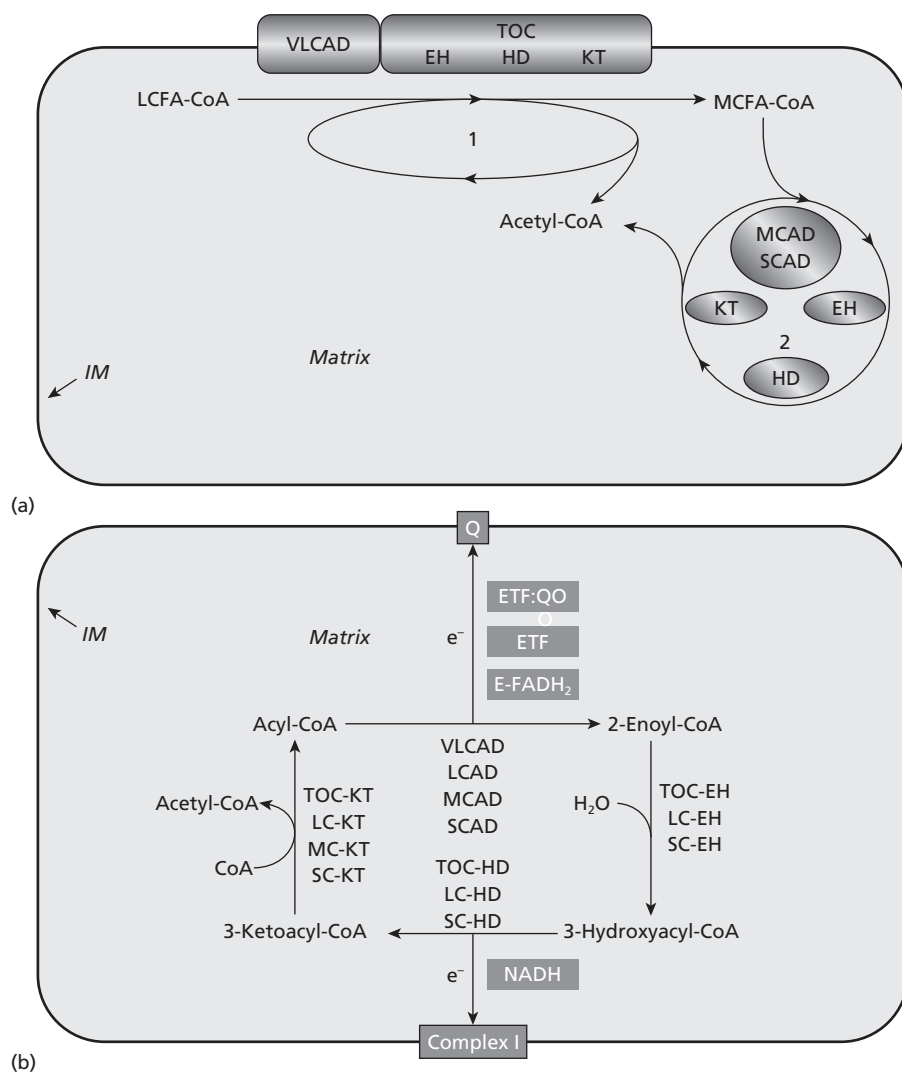
Unlike medium-chain or short-chain fatty acids, long-chain fatty acids cannot directly enter the mitochondria. They must first be activated into a CoA thioester and then transported into the mitochondria by a carnitine-dependent shuttle (Fig. 4) [11]. On the cytosolic face of the outer mitochondrial membrane, acyl-CoA synthetase initially synthesizes an acyl-adenylate intermediate and then forms the acyl-CoA thioester, with the concomitant degradation of one ATP into AMP and pyrophosphate. Carnitine palmitoyl transferase I (CPT I), which is located on the cytosolic face of the outer mitochondrial membrane, next transfers the acyl group from CoA to carnitine (Fig. 4). The acyl-carnitine formed is then translocated across the inner mitochondrial membrane by carnitine acyl-carnitine translocase, in exchange for free carnitine. Finally, carnitine palmitoyl transferase II, which is located on the matrix side of the inner membrane, reforms the long-chain fatty acyl-CoA derivative inside the mitochondrial matrix (Fig. 4).

**Fig. 3** Mitochondrial import of proteins. The mitochondrial proteins, which are encoded by nuclear DNA, are synthesized by cytosolic ribosomes and are imported cotranslationally or posttranslationally into the mitochondria. In the latter case, cytosolic chaperones (hsp) maintain the polypeptide in an import-competent form. All imported polypeptides cross the mitochondrial outer membrane (OM) through the translocase of outer membrane 40 (Tom40) complex. Import routes then differ with the destination of the polypeptide. (1) Polypeptides that are destined to form  $\beta$ -barrel proteins in the OM are brought by small Tim (translocase of the inner membrane) proteins (Tim9 and Tim10) to the sorting and assembly machinery (SAM) complex, which inserts these proteins into the OM. (2) Polypeptides that are destined to form multispanning proteins in the inner membrane (IM) are brought by the same small Tim proteins (Tim9 and Tim10) to the translocase of inner membrane 22 (Tim22) complex. Tim22, which is also called the 'twin translocase' on account of its two pores, inserts these proteins into the IM. (3) Finally, polypeptides that are destined for the mitochondrial matrix usually contain an N-terminal, positively charged presequence. They cross the inner membrane through the Tim23 complex (also called the 'presequence translocase of the inner membrane'). Their mitochondrial import is driven both by the membrane potential ( $\Delta\psi$ ) and by an ATP-driven pulling mechanism mediated by mitochondrial hsp70 (mtHsp70), which is anchored to Tim44. Mitochondrial processing peptidase (MPP) then removes the presequence in the matrix.



**Fig. 4** Activation and mitochondrial uptake of long-chain fatty acids. (1) On the cytosolic face of the mitochondrial outer membrane (OM), the long-chain fatty acid (LCFA) is transformed by acyl-CoA synthetase into acyl-CoA. (2) Carnitine palmitoyl transferase I (CPT I), which is located on the cytosolic face of the outer membrane, transfers the acyl group from CoA to carnitine. It is unclear whether the acyl-carnitine formed is released directly into the intermembrane space by CPT I or whether it is first released outside and then crosses the outer membrane. (3) The acyl-carnitine derivative is translocated across the mitochondrial inner membrane (IM) by carnitine acyl-carnitine translocase (CACT) in exchange for free carnitine. (4) Carnitine palmitoyl transferase II (CPT II), which is located on the matrix side of the inner membrane, then reforms acyl-CoA inside the mitochondrial matrix.





**Fig. 5** Mitochondrial  $\beta$ -oxidation of long-chain fatty acids. (a) Successive action of membrane-bound and then soluble  $\beta$ -oxidation enzymes. (1) The saturated, long-chain fatty acyl-CoA (LCFA-CoA) is shortened to a medium-chain fatty acyl-CoA (MCFA-CoA) by the membrane-bound, very-long-chain acyl-CoA dehydrogenase (VLCAD) and the membrane-bound, trifunctional  $\beta$ -oxidation complex (TOC), which has enoyl-CoA hydratase (EH), 3-hydroxyacyl-CoA dehydrogenase (HD) and 3-ketoacyl-CoA thiolase (KT) activities. (2) The MCFA-CoA is then further split into its acetyl-CoA subunits by soluble matrix enzymes, including medium-chain and short-chain acyl-CoA dehydrogenases (MCAD and SCAD) and soluble EH, HD and KT enzymes specific for long-chain (LC), medium-chain (MC) and short-chain (SC) fatty acids. (b).  $\beta$ -Oxidation intermediates and fate of electrons. The  $\beta$ -oxidation cycle successively involves a first dehydrogenation, a hydration step, a second dehydrogenation and, finally, thiolysis, which releases acetyl-CoA. Electrons coming from acyl-CoA dehydrogenases are transferred to an enzyme-bound FAD, thus forming E-FADH<sub>2</sub>, and are then shuttled by electron transfer flavoprotein (ETF) and ETF-ubiquinone oxidoreductase (ETF:QO), to the ubiquinone (Q) of the respiratory chain, thus forming ubiquinol. Electrons coming from 3-hydroxyacyl-CoA dehydrogenases are transferred to NAD<sup>+</sup>, thus forming NADH, which feeds electrons into complex 1 of the respiratory chain.

Mitochondrial  $\beta$ -oxidation progressively shortens the acyl-CoA by two carbon units at each cycle (Fig. 5) [11]. Each  $\beta$ -oxidation cycle involves a first dehydrogenation leading to *trans*-2-enoyl-CoA, a hydration step forming L-3-hydroxyacyl-CoA, a second dehydrogenation leading to 3-ketoacyl-CoA and, finally, thiolytic cleavage by CoA, releasing acetyl-CoA and an acyl-CoA shortened by two carbon atoms.

First membrane-bound and then soluble enzymes successively shorten a saturated long-chain fatty acyl-CoA (Fig. 5) [12]. The membrane-bound, very-long-chain acyl-CoA dehydrogenase (VLCAD) is also very active with long-chain fatty acids and mediates the first dehydrogenation step of the  $\beta$ -oxidation cycle. The membrane-bound, trifunctional  $\beta$ -oxidation complex (TOC) then completes the  $\beta$ -oxidation cycle through its enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities. This membrane-bound system (VLCAD and TOC) may shorten the saturated long-chain fatty acid into a medium-chain fatty acid (Fig. 5). Further shortening is then mediated by matrix enzymes, including medium-

chain and short-chain acyl-CoA dehydrogenases (MCAD and SCAD) and soluble enoyl hydratases, 3-hydroxyacyl-CoA dehydrogenases and 3-ketoacyl-CoA thiolases specific for long-, medium- or short-chain fatty acids (Fig. 5) [11,12].

Owing to the high activity of the membrane-bound system, the soluble, long-chain acyl-CoA dehydrogenase (LCAD) is redundant for the  $\beta$ -oxidation of saturated long-chain fatty acids. However, LCAD is involved in the  $\beta$ -oxidation of unsaturated long-chain fatty acids, thus compensating for the poor activity of VLCAD for unsaturated substrates [12].

The two main steps regulating the  $\beta$ -oxidation flux are carnitine palmitoyl transferase I, which modulates the entry of long-chain fatty acids into the mitochondria, and acyl-CoA dehydrogenases, whose activities are relatively sluggish compared with the high activities of other  $\beta$ -oxidation enzymes [13].

Each  $\beta$ -oxidation cycle removes four electrons. The two electrons removed by acyl-CoA dehydrogenases transiently form an enzyme-bound E-FADH<sub>2</sub>. These electrons are then shuttled by electron transfer flavoprotein (ETF) and ETF-ubiquinone

oxidoreductase (ETF:QO) to the ubiquinone (Q) of the respiratory chain close to complex III, thus forming ubiquinol (Fig. 5). In contrast, the electrons removed by 3-hydroxyacyl-CoA dehydrogenases are transferred to  $\text{NAD}^+$  to form nicotinamide adenine dinucleotide (NADH), which feeds electrons into complex I of the respiratory chain (Fig. 5). As discussed below, electrons coming from E-FADH<sub>2</sub> will produce about two ATP molecules, while those coming from NADH will form about three ATP molecules [14]. Therefore, each round of mitochondrial  $\beta$ -oxidation eventually leads to about five ATP molecules and one molecule of acetyl-CoA. The latter can either condense into ketone bodies, as discussed below, or may eventually undergo the tricarboxylic acid cycle to generate much more energy.

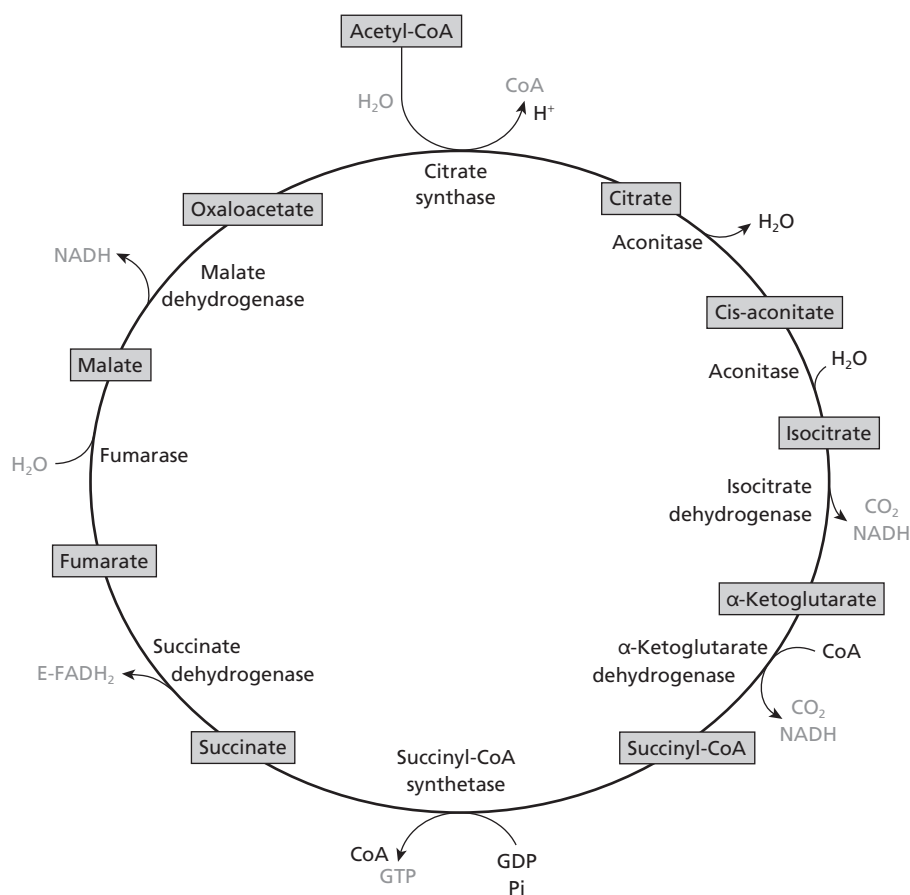
### Tricarboxylic acid cycle

The final oxidation of acetyl-CoA by the tricarboxylic acid cycle (also termed the 'citric acid cycle', starts with the condensation of acetyl-CoA with oxaloacetate to form citrate (Fig. 6) [14]. This reaction is carried out by citrate synthase and involves the formation a citryl-CoA intermediate, which is then hydrolysed into CoA and citrate.

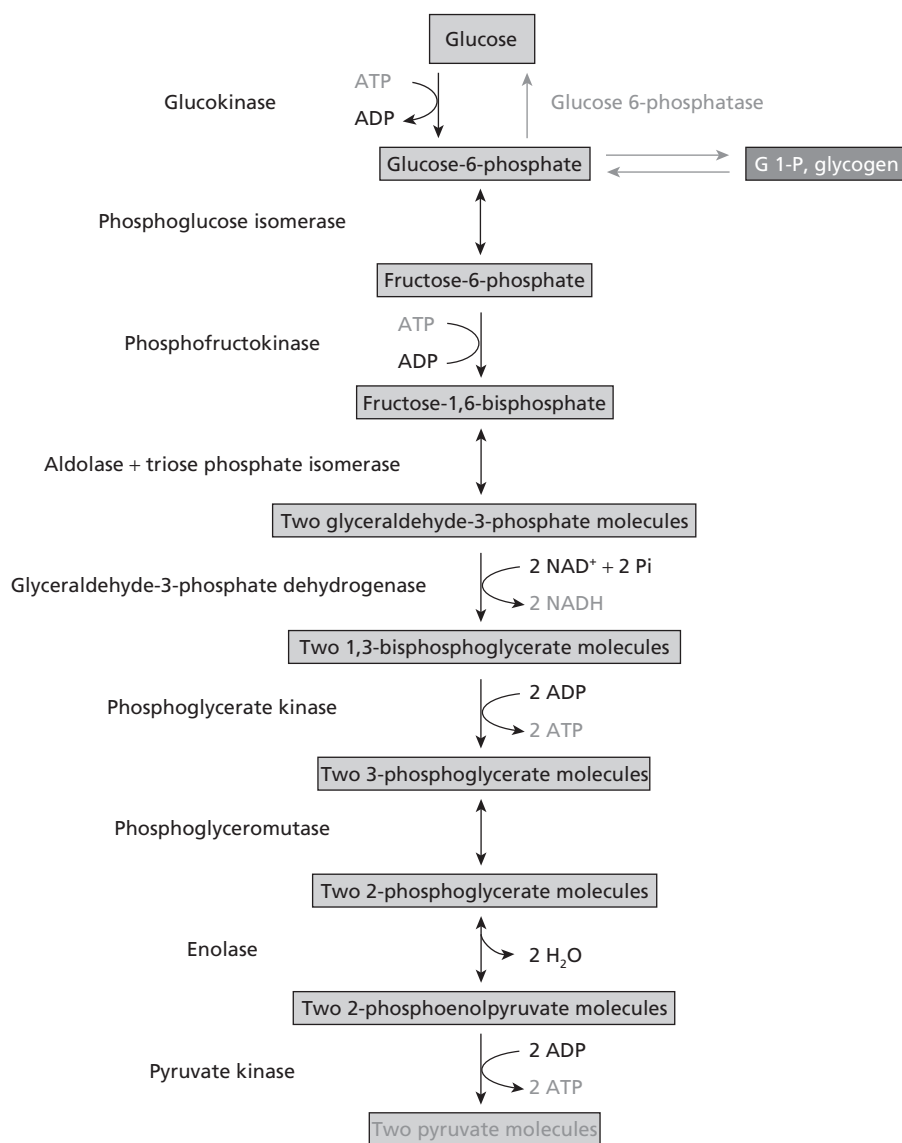
The tricarboxylic acid cycle then proceeds with the isomerization of citrate into isocitrate (mediated by aconitase), the oxidative

decarboxylation of isocitrate into  $\text{CO}_2$  and  $\alpha$ -ketoglutarate (mediated by isocitrate dehydrogenase), and the CoA-supported oxidative decarboxylation of  $\alpha$ -ketoglutarate into  $\text{CO}_2$  and succinyl CoA (mediated by the  $\alpha$ -ketoglutarate dehydrogenase complex). The cycle further proceeds with the cleavage of the thioester bond of succinyl-CoA to form succinate and one high-energy guanosine 5'-triphosphate (GTP) (mediated by the reversibly acting, succinyl-CoA synthetase). Finally, the cycle ends up with three steps resembling those involved in the  $\beta$ -oxidation process, with the dehydrogenation of succinate into fumarate (mediated by succinate dehydrogenase), the hydration of fumarate into malate (mediated by fumarase) and the dehydrogenation of malate into oxaloacetate (mediated by malate dehydrogenase). The overall stoichiometry of the tricarboxylic acid cycle is:  $\text{acetyl-CoA} + 2 \text{H}_2\text{O} + 3 \text{NAD}^+ + \text{FAD} + \text{GDP} + \text{P}_i \rightarrow 2 \text{CO}_2 + 3 \text{NADH} + \text{FADH}_2 + \text{GTP} + 2 \text{H}^+ + \text{CoA}$ . As each of the three NADH molecules will eventually give about three ATP molecules and the single FADH<sub>2</sub> will form about two ATP molecules, the global energy yield of one citric acid cycle is about 11 ATP molecules, as well as one energy-rich GTP molecule, which can be used as such or can generate one more ATP molecule [14].

It is noteworthy that molecular oxygen is not immediately used in this cycle nor in the  $\beta$ -oxidation cycle. The oxygen



**Fig. 6** Tricarboxylic acid cycle. The final oxidation of the acetate moiety of acetyl-CoA by the tricarboxylic acid cycle produces one molecule of energy-rich GTP, three molecules of NADH and one molecule of enzyme-bound FADH<sub>2</sub> within succinate dehydrogenase (E-FADH<sub>2</sub>). NADH and E-FADH<sub>2</sub> then transfer their electrons to the respiratory chain to produce ATP.



**Fig. 7** Glycolysis. The glycolysis of glucose in the cytoplasm forms two molecules of pyruvate. Unless the generated NADH and pyruvate are further oxidized in mitochondria, glycolysis itself only generates two net molecules of ATP.

atoms, which end up in  $\text{CO}_2$ , come from water. However, as discussed below, the electrons, which are passed by NADH and  $\text{FADH}_2$  to the respiratory chain, end up in molecular oxygen to form water. This transfer of electrons regenerates the oxidized cofactors ( $\text{NAD}^+$  and FAD), which are necessary to sustain  $\beta$ -oxidation and the tricarboxylic acid cycle. Thus, even though molecular oxygen is not involved in the tricarboxylic acid cycle, oxygen is mandatory at the next step, explaining why the tricarboxylic acid cycle can only function in the presence of oxygen. In the absence of oxygen, the only metabolic pathway that can transiently provide some feeble energy is glycolysis.

### Cytosolic glycolysis and the mitochondrial oxidation of pyruvate

Glycolysis is defined as the process that converts glucose into pyruvate [14]. This process, which occurs in the cytoplasm,

starts with the phosphorylation of glucose into glucose-6-phosphate. This reaction is catalysed by glucokinase in the liver and hexokinase in other organs (Fig. 7). Glucose-6-phosphate is a branching point in glucose metabolism. Its reversible isomerization to glucose-1-phosphate can lead to UDP-glucose formation and glycogen synthesis, whereas its reversible isomerization into fructose-6-phosphate can lead to glycolysis. The first committed step in glycolysis is the irreversible phosphorylation of fructose-6-phosphate into fructose-1,6-bisphosphate by phosphofructokinase. After a series of enzymatic reactions detailed in Figure 7, including the reduction of  $\text{NAD}^+$  into NADH by glyceraldehyde-3-phosphate dehydrogenase, glycolysis ends up with the irreversible transformation of phosphoenol pyruvate into pyruvate, a reaction catalysed by pyruvate kinase (Fig. 7). Both phosphofructokinase and the liver-type pyruvate kinase are tightly regulated (through allosteric and phosphorylation events) to prevent the liver from consuming glucose when glucose is more

urgently needed by brain or muscle [14]. The net stoichiometry of glycolysis is:  $\text{glucose} + 2 \text{NAD}^+ + 2 \text{ADP} + 2 \text{Pi} \rightarrow 2 \text{pyruvate} + 2 \text{NADH} + 2 \text{H}^+ + 2 \text{ATP} + 2 \text{H}_2\text{O}$  [14].

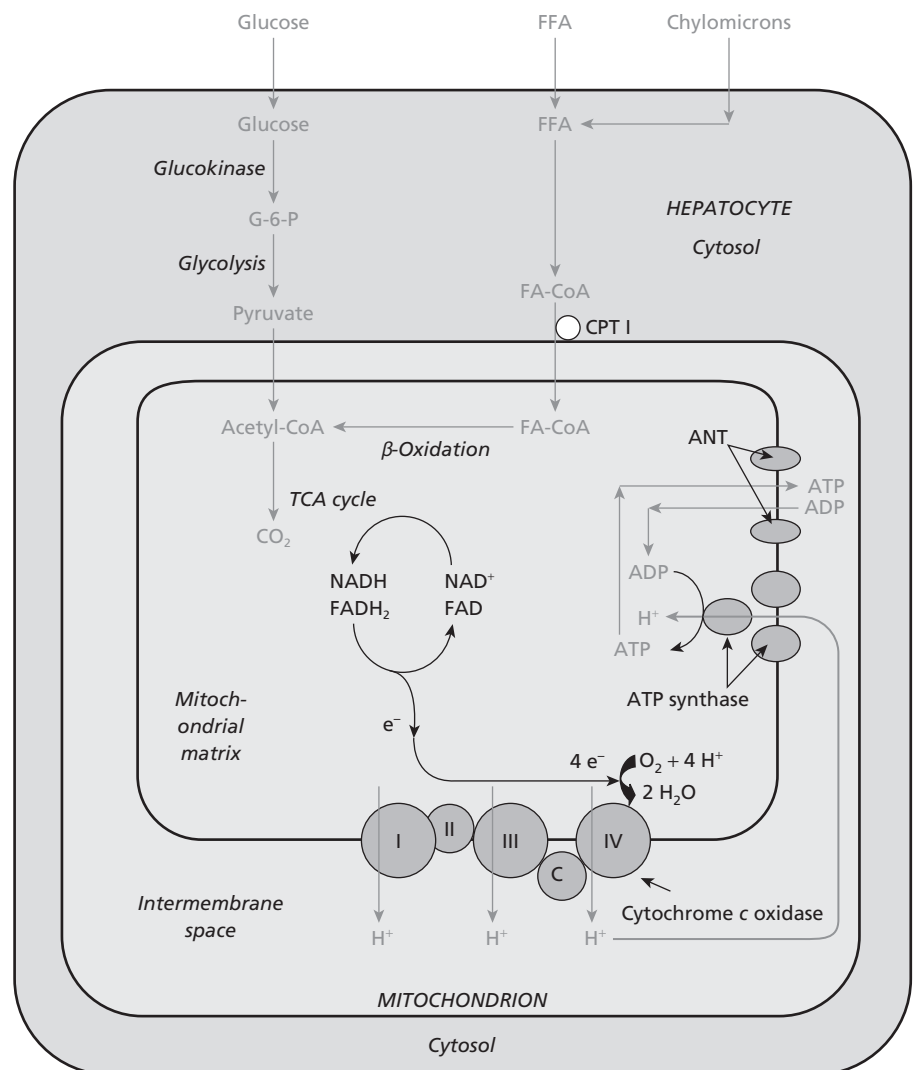
Glycolysis cannot proceed unless the formed NADH is reoxidized into the  $\text{NAD}^+$  required by glyceraldehyde-3-phosphate dehydrogenase. When there is a transient incapacity of mitochondria to reoxidize the formed NADH (for example, during strenuous muscular exercise or during tissue ischaemia), some transient relief can be obtained from the reversible reaction catalysed by lactate dehydrogenase:  $\text{NADH} + \text{H}^+ + \text{pyruvate} \rightarrow \text{NAD}^+ + \text{lactate}$ . The regeneration of  $\text{NAD}^+$  allows glycolysis to continue, while the formed lactate can be oxidized at later times in the same organ (e.g. in the resting muscle) or in other organs, such as the liver. In the meantime, however, the energy yield is only two molecules of ATP for one molecule of glucose consumed, so that anaerobic glycolysis is a most wasteful way of generating energy.

However, under normal aerobic conditions, mitochondria reoxidize the NADH formed by glycolysis. Because the inner

mitochondrial membrane is impermeable to NADH, its electrons rather than NADH are transferred to mitochondria. This can be achieved by two mitochondrial shuttles: the malate–aspartate or the glycerol phosphate shuttles [14]. In the cytosol, NADH regenerates  $\text{NAD}^+$  by transferring its electrons to either oxaloacetate or dihydroxyacetone phosphate, which then diffuse into the mitochondria, where they release their electrons to regenerate either NADH (in the malate–aspartate shuttle) or an enzyme-bound  $\text{FADH}_2$  molecule (in the glycerol phosphate shuttle) [14]. These reduced cofactors then form ATP through oxidative phosphorylation, as discussed below.

Under normal aerobic conditions, mitochondria also oxidize the pyruvate (or lactate) formed by glycolysis (Fig. 8). Mitochondrial carriers transport pyruvate or lactate into the mitochondria, where the pyruvate dehydrogenase complex catalyses the reaction:  $\text{pyruvate} + \text{NAD}^+ + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{NADH}$ . The oxidation of this acetyl-CoA in the tricarboxylic acid cycle will then generate considerable energy. Whereas the anaerobic glycolysis of glucose into pyruvate and lactate only

**Fig. 8** Oxidative metabolism and energy production by mitochondria. The oxidation of pyruvate and free fatty acids (FFA) inside mitochondria produces NADH and  $\text{FADH}_2$ , which transfer their electrons to the mitochondrial respiratory chain. The flow of electrons in complexes 1, 3 and 4 is coupled with the extrusion of protons from the mitochondrial matrix into the intermembrane space. When energy is needed, these protons re-enter the matrix through ATP synthase to generate ATP from ADP. The adenine nucleotide translocator (ANT) then exchanges the formed ATP for cytosolic ADP. G-6-P, glucose 6-phosphate; LCFA-CoA, long-chain fatty acyl-CoA; CPT I, carnitine palmitoyl transferase I; TCA cycle, tricarboxylic acid cycle; c, cytochrome c.



forms two ATP molecules, the complete aerobic oxidation of glucose can provide up to 38 ATP molecules, thanks to the oxidative phosphorylation system [14].

## Oxidative phosphorylation system

Oxidative phosphorylation is the process whereby the electrons stored in NADH or enzyme-bound  $\text{FADH}_2$  are reacted with oxygen to form water, while part of the energy released by this exergonic reaction is harnessed to form ATP. To achieve this aim, electrons are first transferred through different respiratory chain complexes, where the electron transfer potential is converted, step by step, into an electrochemical proton gradient, which is finally converted into a phosphate transfer potential by forming energy-rich ATP molecules [14]. An analogy may be the pumping up of water behind a dam by a thermal engine, and then the opening of water valves allowing the hydrostatic flow of water into a hydroelectric turbine that generates electricity used to charge small batteries.

A total of five complexes constitute the oxidative phosphorylation system [15]. Complexes 1–4 constitute the respiratory chain, while complex 5 is ATP synthase. Depending on their original sources (NADH or enzyme-bound  $\text{FADH}_2$ ), electrons enter the respiratory chain at different sites.

- The two electrons coming from NADH are transferred to complex 1 (also termed ‘NADH-ubiquinone oxidoreductase’ or ‘NADH dehydrogenase’). Within this large, L-shaped complex, electrons are successively transferred to one flavin mononucleotide (FMN) and several iron–sulphur clusters to be finally donated to ubiquinone (Q), thus forming ubiquinol ( $\text{QH}_2$ ). Concomitantly, four protons are pumped from the mitochondrial matrix into the intermembrane space. Therefore, the stoichiometry of the reaction catalysed by complex I is:  $\text{NADH} + 5 \text{H}_{\text{in}}^+ + \text{Q} \rightarrow \text{NAD}^+ + 4 \text{H}_{\text{out}}^+ + \text{QH}_2$  [16].
- The two electrons coming from the enzyme-bound  $\text{FADH}_2$  of succinate dehydrogenase are given to complex 2. Unlike complexes 1, 3 or 4, complex 2 does not translocate protons from the mitochondrial matrix into the intermembrane space. It only transfers two electrons to ubiquinone, thus forming ubiquinol.
- Finally, the electrons coming from the enzyme-bound  $\text{FADH}_2$  of acyl-CoA dehydrogenases are directly transferred by ETF and ETF:QO to ubiquinone, thus forming ubiquinol. Thus, whatever their initial site of entry (complex 1, complex 2 or ubiquinone), electrons end up in ubiquinol.

Q and  $\text{QH}_2$  are highly mobile molecules within the inner mitochondrial membrane, allowing  $\text{QH}_2$  to transfer its two electrons to complex 3 (also termed ‘ubiquinol:cytochrome *c* oxidoreductase’ or ‘cytochrome *bc\_1*’). The redox active sites of complex 3 include a cytochrome *b* with two haems (b-562 and b-566), an iron–sulphur protein (ISP) and a membrane-anchored cytochrome *c\_1*. The ‘Q cycle’ theory postulates that complex 3 contains two sites for  $\text{Q}/\text{Q}^-/\text{QH}_2$ : an outside  $\text{Q}_o$  site located in close proximity to the intermembrane space and an inner  $\text{Q}_i$  site located in close proximity to the matrix (Fig. 9) [15]. At the

outside  $\text{Q}_o$  site, ubiquinol would produce ubiquinone by releasing its two protons into the intermembrane space and by giving one electron to the ISP (to be given next to cytochrome *c\_1* and then cytochrome *c*) and the second electron to cytochrome *b* [17]. Cytochrome *b* would then recycle this electron in the  $\text{Q}_i$  site, as explained in the legend to Figure 9. Another theory holds that ubiquinol donates its two electrons to cytochrome *b*, which then gives them to the ISP, en route for cytochrome *c\_1* and cytochrome *c* (Fig. 9) [18]. Whatever the path(s) of electrons, the net stoichiometry of the reaction catalysed by complex 3 is:  $\text{QH}_2 + 2 \text{oxidized cytochrome } c (\text{Fe}^{3+}) + 2 \text{H}_{\text{in}}^+ \rightarrow \text{Q} + 2 \text{reduced cytochrome } c (\text{Fe}^{2+}) + 4 \text{H}_{\text{out}}^+$  [15].

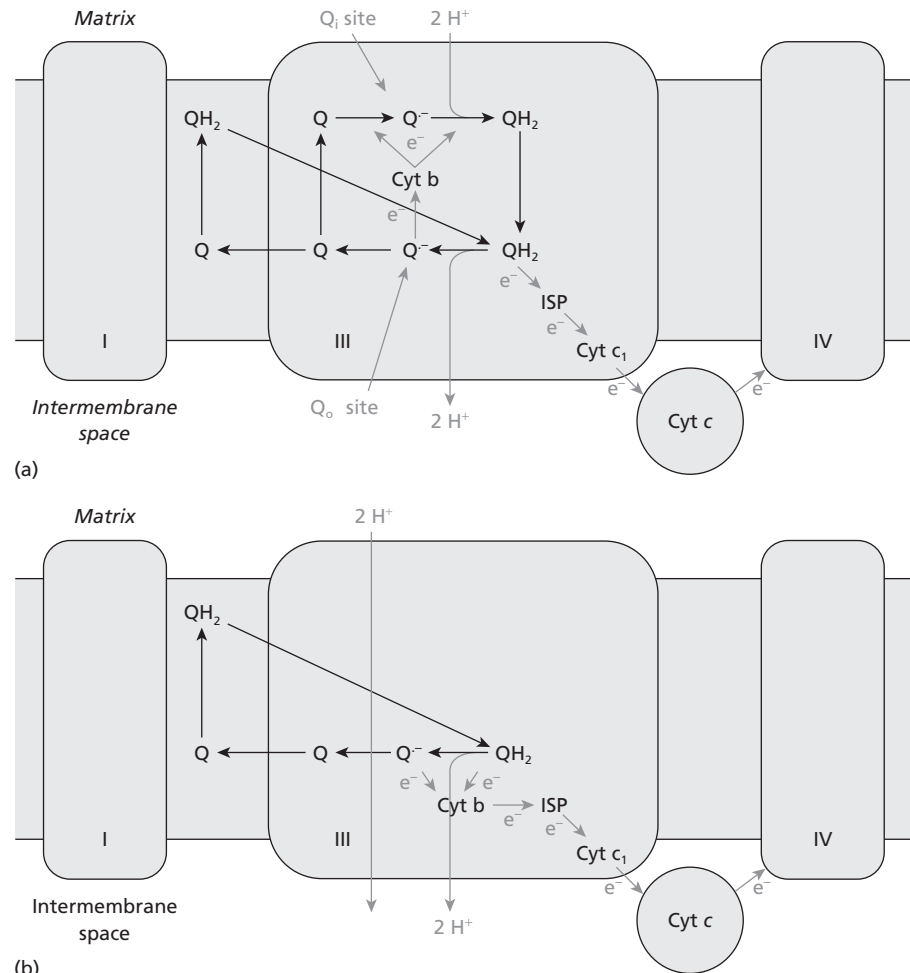
Four reduced cytochrome *c* molecules finally transfer four electrons to complex 4 (cytochrome *c* oxidase), where the four electrons are successively, but quickly, reacted with an oxygen molecule, which is held in a tight cage so that partially reduced oxygen species are not released, but only water is safely formed [15]. The generation of water also consumes four protons, which are taken from the matrix side. Concomitantly, complex 4 transports four protons from the mitochondrial matrix into the intermembrane space. Therefore, the stoichiometry of the reaction catalysed by complex 4 is:  $4 \text{reduced cytochrome } c (\text{Fe}^{2+}) + \text{O}_2 + 8 \text{H}_{\text{in}}^+ \rightarrow 4 \text{oxidized cytochrome } c (\text{Fe}^{3+}) + 2 \text{H}_2\text{O} + 4 \text{H}_{\text{out}}^+$  [15].

Thus, the transfer of electrons through complexes 1, 3 and 4 of the respiratory chain is coupled with the extrusion of protons from the mitochondrial matrix into the mitochondrial intermembrane space. This creates a large electrochemical potential across the inner membrane, thus creating a reservoir of latent, potential energy (Fig. 8).

When energy is needed, the increase in ADP stimulates the re-entry of protons through ATP synthase (also called ‘ $\text{F}_1\text{F}_0$ -ATPase’, ‘ $\text{H}^+$ -ATPase’ or ‘complex 5’), and the energy that is liberated by this re-entry is harnessed in the synthesis of ATP from ADP. The ATP formed is then extruded from mitochondria by the adenine nucleotide translocator in exchange for cytosolic ADP (Fig. 8). ATP is then used by all the cellular processes that require energy (e.g. maintenance of transmembrane ion gradients, anabolic syntheses, cell motility, muscular contraction).

ATP synthase is a wonderful biological engine (Fig. 10) [19,20]. Its transmembrane  $\text{F}_0$  portion contains a non-rotating moiety consisting of the a subunits and a rotor made of a dozen or so c subunits (c-ring rotor). The re-entry of protons through a channel located at the junction of the a subunits and the c-ring triggers the rotation of the c-ring and the attached  $\gamma$  stalk. The  $\gamma$  stalk rotates within a cap made of three pairs of alternating  $\alpha$  and  $\beta$  subunits ( $\alpha_3\beta_3$  cap), which is kept immobile by the a/b<sub>2</sub>/δ stator [20]. The rotating stalk is asymmetrical. When the stalk rotates within the  $\alpha_3\beta_3$  cap, it causes rhythmic deformations of the catalytic  $\beta$  subunits, which may alternatively tighten around ADP and  $\text{P}_i$ , close to form ATP and fully open to release ATP. As there are three catalytic sites, one 360° rotation of the  $\gamma$  stalk gives rise to three ATP molecules [19].

**Fig. 9** Current hypotheses for the flow of electrons within complex 3. (a) The classical 'Q cycle' theory postulates that complex 3 contains two sites for ubiquinone (Q) and its redox partners, the ubisemiquinone anion radical ( $Q^{\cdot-}$ ) and ubiquinol ( $QH_2$ ). The outside  $Q_o$  site would be close to the intermembrane space, and the inner  $Q_i$  site close to the matrix. Two successive ubiquinol molecules coming from other complexes may enter the outside  $Q_o$  site of complex 3, where each ubiquinol molecule would produce ubiquinone by releasing its two protons into the intermembrane space. In the process, ubiquinol would give one electron to the iron-sulphur protein (ISP), which then transfers this electron to cytochrome  $c_1$ , which in turn gives it to cytochrome  $c$ . The semiubiquinone anion radical formed from ubiquinol would then give its electron to cytochrome  $b$  (Cyt  $b$ ), thus forming ubiquinone, which would be returned to the region between complex 1 and complex 3. Concomitantly, cytochrome  $b$  would recycle the electrons that it receives at the  $Q_o$  site by sending them to the  $Q_i$  site. At this  $Q_i$  site, cytochrome  $b$  would first give one electron to  $Q$ , thus forming  $Q^{\cdot-}$ , and the next electron to  $Q^{\cdot-}$ , thus forming  $QH_2$ . The formed ubiquinol would then move to the  $Q_o$  site to be oxidized in exchange for ubiquinone coming from the  $Q_i$  site to be reduced. Thus, according to this scheme, for two ubiquinol molecules releasing their protons through their oxidation at the  $Q_o$  site, one molecule of ubiquinol would be regenerated at the  $Q_i$  site. (b) Another theory holds that ubiquinol instead donates its two electrons to cytochrome  $b$ , which then gives them to the iron sulphur protein (ISP), en route for cytochrome  $c_1$ , and cytochrome  $c$ .



Because the driving force for ATP synthesis is the re-entry of protons, and because the flow of electrons along the respiratory chain extrudes protons at three different sites, the number of ATP molecules that can be synthesized differs with the site of electron entry into the chain. The electrons that are transferred from NADH cause the extrusion of protons at complex 1, complex 3 and complex 4 and can lead to the synthesis of about three ATP molecules for one molecule of NADH reoxidized [14]. In contrast, the electrons that are transferred by enzyme-bound  $FADH_2$  bypass complex 1 and only permit the synthesis of about two ATP molecules for one  $FADH_2$  reoxidized [14].

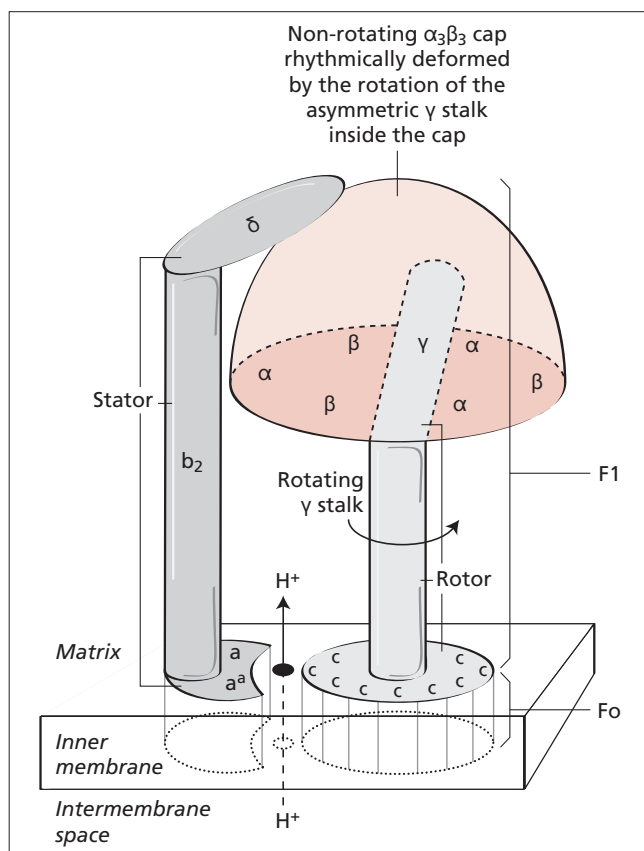
Interestingly, ATP synthase is a reversible machine. When the mitochondrial membrane potential is low, for example during ischaemia, ATP synthase functions in the reverse mode. Its ATPase activity consumes the meagre amounts of ATP formed by glycolysis to pump protons into the intermembrane space and partially to restore the mitochondrial membrane potential, which is needed to maintain mitochondrial protein import and mitochondrial viability. This ATP-depleting effect can be

prevented by the binding of a matricial protein termed the inhibitor of  $F_1F_0$ -ATPase ( $IF_1$ ) [21]. When the membrane potential is low (e.g. during ischaemia) and when anaerobic glycolysis causes extensive lactate formation and matrix acidification,  $IF_1$  then binds to the  $F_1$  moiety of ATP synthase to inhibit its ATPase activity. This attenuates the selfish use of cell ATP by deenergized mitochondria.

Except for these extreme circumstances, when mitochondria consume cell ATP for their own needs, mitochondria instead provide the cell with energy.

## Regulation of fuel oxidation by energy demands

Mitochondria are thrifty organelles. They only burn fuels inasmuch as fuel oxidation is needed to satisfy the energy demands of the cell. This balance is achieved through several mechanisms, including built-in, autoregulatory mechanisms adapting fuel oxidation (and thus ATP generation) to ATP consumption.



**Fig. 10** ATP synthase. The transmembrane  $F_0$  portion of ATP synthase contains a non-rotating moiety (the  $a$  subunits) and a membrane-embedded rotor made of a dozen  $c$  subunits (the  $c$ -ring rotor). The re-entry of protons at the junction of the  $a$  subunits and the  $c$ -ring rotor triggers the rotation of the  $c$ -ring rotor and the attached  $\gamma$  rod, which rotates within an immobile cap made of three pairs of alternating  $\alpha$  and  $\beta$  subunits ( $\alpha_3\beta_3$  cap). The cap is prevented from rotating by the  $a/b_2/\delta$  stator. The rotating  $\gamma$  stalk is asymmetrical (depicted illustratively as a bend on the figure). Its rotation within the  $\alpha_3\beta_3$  cap causes rhythmic deformations of the catalytic  $\beta$  subunits, which may successively tighten around ADP and  $P_i$ , close to form ATP and fully open to release ATP. As there are three catalytic sites, one  $360^\circ$  rotation of the  $\gamma$  stalk produces three ATP molecules.

When energy demands are high, and ADP is high, a massive re-entry of protons into the matrix through ATP synthase decreases the mitochondrial membrane potential. This unleashes the flow of electrons in the respiratory chain, with three consequences. First, the increased flow of electrons increases the rate of proton extrusion by the respiratory chain, thus allowing the continued re-entry of protons through ATP synthase to sustain high rates of ATP generation. Second, the increased flow of electrons ends up in cytochrome  $c$  oxidase, thus increasing oxygen consumption. Finally, the increased rate of respiration is associated with an increased reoxidation of NADH and  $FADH_2$  into  $NAD^+$  and  $FAD$ , which are required to drive the oxidation of glucose, pyruvate, acyl-CoA and acetyl-CoA.

In contrast, when little energy is needed by the cell, ATP synthase is barely active and only translocates a few protons back into the matrix. The high mitochondrial membrane potential blocks the extrusion of protons into the matrix. Electrons are stuck in the respiratory chain. Oxygen consumption is low. The limited reoxidation of NADH into the  $NAD^+$  required for mitochondrial  $\beta$ -oxidation and the tricarboxylic acid cycle may limit the capacity of mitochondria to oxidize fat and pyruvate. Thus, the tricarboxylic acid cycle and oxidative phosphorylation system have built-in mechanisms that automatically adapt fuel oxidation to energy consumption.

This automatic adaptation is further tuned by the regulatory effects of ATP on several critical enzymatic steps [14]. Indeed, the activities of pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase are modulated by ATP levels, in order to increase fuel oxidation when ATP is low and, instead, to prevent fuel oxidation when ATP is high [14].

A last level of regulation involves the effects of nuclear genes on mitochondrial function and biogenesis.

## Nuclear control of mitochondrial function and biogenesis

Both mitochondrial function and mitochondrial biogenesis are controlled by nuclear genes [22]. When mitochondrial function is insufficient to sustain the cellular drain of ATP, cell ADP increases. Adenylate kinase then converts two ADP molecules into one ATP molecule and one AMP molecule [23]. The increase in AMP is sensed by AMP-activated protein kinase (AMPK), which turns off ATP-consuming pathways and switches on ATP-producing catabolic pathways [23]. In particular, AMPK phosphorylates and inactivates acetyl-CoA carboxylase, thus decreasing the synthesis of malonyl-CoA. This prevents the inhibition of CPT 1 by malonyl-CoA, thus allowing the translocation of long-chain fatty acyl-CoA into the mitochondria and extensive  $\beta$ -oxidation [23]. Finally, AMPK increases several mitochondrial oxidative enzymes, such as succinate dehydrogenase, citrate synthase and cytochrome  $c$  [24,25].

Several other factors may also modulate mitochondrial function and biogenesis in the liver, although their roles have mostly been studied in skeletal muscles, whose energy requirements vary widely with the degree of physical activity. These other factors include the  $Ca^{2+}$ /calmodulin-dependent protein kinase (CaMK), the peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 (PGC-1) and nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2). In muscles, it has been shown that CaMK induces the expression of PGC-1, which itself induces the expression of NRF-1 and NRF-2. NRF-1 and NRF-2 increase the synthesis of nuclear DNA-encoded polypeptides of the respiratory chain and also induce mtTFA [22,26]. PGC-1 binds to, and coactivates, the transcriptional function of NRF-1 on the promoter for mtTFA, which increases the transcription and replication of mtDNA [26]. Finally, PGC-1 binds to, and cooperates with, peroxisome

proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ) in the transcriptional activation of nuclear genes encoding peroxisomal and mitochondrial  $\beta$ -oxidation enzymes, including CPT I and medium-chain acyl-CoA dehydrogenase [27]. As a consequence of these concerted actions on both nuclear genes and mtDNA genes, PGC-1 increases both the number of cardiac mitochondria and also their individual capacity to oxidize fat and to generate energy through oxidative phosphorylation [28]. Interestingly, both the AMPK- and the CaMK-mediated signals seem to act coordinately, as AMPK was required for energy deprivation-induced increases in the expression of CaMK and PGC-1 [29]. Together with the immediate regulations described in the preceding section, these nuclear changes may allow cells to increase or decrease fuel consumption depending on the energy demands of the cell.

Yet a last type of regulation concerns the different use of energetic fuels under fasted, fed or overfed conditions.

### **Different use of fuels by hepatic mitochondria in fasted, fed or overfed conditions** (see also Chapter 13)

The handling of fuels by hepatic mitochondria is regulated in order to deliver ketone bodies and glucose to other organs when food is not available, but to store energy as glycogen and fat after meals.

In the fasting state, low insulin levels allow the massive release of free fatty acids from adipose tissue [30]. During fasting conditions, hepatic malonyl-CoA levels are low, allowing extensive mitochondrial import of long-chain free fatty acids, and thus active fatty acid  $\beta$ -oxidation. However, the acetyl-CoA moieties generated by hepatic mitochondrial  $\beta$ -oxidation are not fully oxidized in the liver, which would not be able to use this enormous amount of energy. Instead, acetyl-CoA moieties condense into ketone bodies (acetoacetate and  $\beta$ -hydroxybutyrate), which are secreted by the liver to be oxidized later in muscles and other peripheral tissues by the tricarboxylic acid cycle, thus sparing glucose [30]. The liver concomitantly provides other organs, such as the brain, with glucose formed from glycogen and gluconeogenesis.

In contrast, after meals, a slight increase in blood glucose increases the release of insulin by pancreatic  $\beta$  cells. In adipocytes, insulin inhibits hormone-sensitive lipase, thus blocking adipose tissue lipolysis. In the liver, glucose inactivates glycogen phosphorylase and activates glycogen synthetase, thus increasing glycogen stores [14]. Furthermore, both insulin and glucose trigger free fatty acid synthesis from glucose [31]. This is due to the posttranslational activation of several enzymes [14] and also to translational regulation [31]. Indeed, glucose activates carbohydrate response element binding protein (ChREBP), with two consequences. First, ChREBP stimulates liver-type pyruvate kinase, thus increasing the glycolysis of glucose into pyruvate [32]. In mitochondria, pyruvate forms acetyl-CoA, which condenses with oxaloacetate to form citrate

that translocates to the cytoplasm. Citrate lyase then catalyses the reaction: citrate + ATP + CoA + H<sub>2</sub>O  $\rightarrow$  acetyl-CoA + oxaloacetate + ADP + P<sub>i</sub>. The acetyl-CoA, which is thus regenerated in the cytosol, is then used by acetyl-CoA carboxylase to form malonyl-CoA, which is required for the elongation of free fatty acids by fatty acid synthase. Second, ChREBP stimulates the transcription of all lipogenic genes, including acetyl-CoA carboxylase, fatty acid synthase and stearoyl-CoA desaturase, thus further increasing hepatic fatty acid and triacylglycerol synthesis [33]. Insulin cooperates with glucose in increasing fatty acid synthesis. Indeed, insulin increases the transcription of sterol regulatory element-binding protein-1c (SREBP-1c), which increases the transcriptional activation of all the enzymes required for the hepatic synthesis of fatty acids [31]. Thus, both glucose via ChREBP and insulin via SREBP-1c can increase the hepatic synthesis of free fatty acids. Furthermore, the malonyl-CoA generated by acetyl-CoA carboxylase also prevents mitochondrial  $\beta$ -oxidation. Malonyl-CoA inhibits CPT I, which controls the entry of long-chain fatty acids into mitochondria [30]. After a carbohydrate meal, high malonyl-CoA levels inhibit CPT I and prevent fatty acid  $\beta$ -oxidation [30]. Free fatty acids are not degraded, but are instead directed towards the formation of triglycerides, which are partly stored in the liver and partly secreted as very-low-density lipoproteins.

Teleologically, these various changes can be viewed as maintaining/increasing fat and glycogen stores when food is plentiful and, in contrast, using up fat and sparing glucose when food is not available. In the past, these changes have provided a satisfactory balance between energy storage and energy use, when high physical activity ensured high rates of fuel oxidation by skeletal muscle mitochondria. Nowadays, however, food intake often exceeds the limited capacity of mitochondria to burn fuels in inactive persons, causing a progressive increase in body fat stores [34].

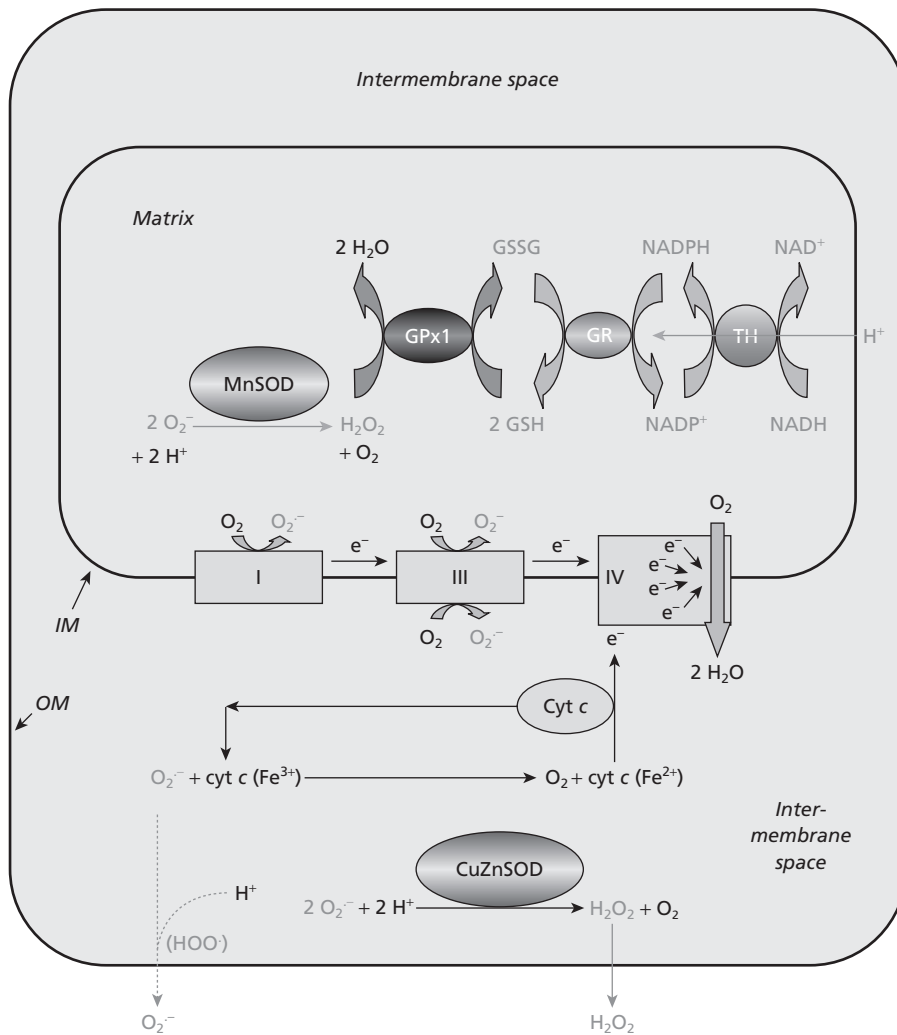
In relatively overfed subjects, the increased hepatic synthesis of free fatty acids causes the accumulation of triglyceride droplets in the cytoplasm of hepatocytes [34]. However, the liver does not enlarge indefinitely. A new equilibrium is reached when the increased synthesis of fat is compensated by increased output pathways, including an increased mitochondrial  $\beta$ -oxidation of fatty acids [34]. Several adaptive changes may allow hepatic mitochondria to oxidize more fat in overfed subjects [34]. A first mechanism could be the increased hepatic concentrations of free fatty acids (FFAs) in these subjects, which may force the entry of FFAs into the mitochondria [34]. A second mechanism may involve changes in CPT I expression and its sensitivity to malonyl-CoA inhibition. CPT I is upregulated in rodent models of diabetes, and its affinity for its physiological inhibitor, malonyl-CoA, is decreased [35,36]. Loss of CPT I inhibition by malonyl-CoA could explain why  $\beta$ -oxidation can increase in plethoric subjects, despite high insulin and malonyl-CoA levels, which would normally block the entry of long-chain fatty acids into mitochondria [34]. An important upstream mediator of these diverse changes may be PPAR- $\alpha$ .

PPAR- $\alpha$  is activated by long-chain free fatty acids and increases the expression of enzymes involved in peroxisomal and mitochondrial  $\beta$ -oxidation, including CPT I [35]. PPAR- $\alpha$  activation also increases uncoupling protein 2 mRNA [37]. This uncoupling protein might allow the re-entry of protons from the intermembrane space into the mitochondrial matrix. This could decrease the mitochondrial membrane potential, unleash the flow of electrons in the respiratory chain, increase mitochondrial respiration and allow better reoxidation of NADH into the NAD<sup>+</sup> required for fatty acid oxidation.

Although these adaptive changes prevent further expansion of hepatic fat stores in overfed subjects, they do not prevent some steatosis. The unsaturated lipids of fat deposits can then be oxidized by the ROS formed by mitochondria, thus triggering lipid peroxidation and oxidative stress [34].

## Formation of reactive oxygen species

The incomplete reduction of oxygen by one, two or three electrons produces ROS. The strategy devised by bacteria and mitochondria to limit ROS formation is initially to store electrons in a form (NADH or E-FADH<sub>2</sub>) that is unreactive with oxygen, then to transport these electrons through a respiratory chain, which is mostly insulated from oxygen, and, finally, quickly to react four electrons with oxygen within cytochrome *c* oxidase to safely produce water. However, the insulation of upstream respiratory complexes from oxygen is not perfect (Fig. 11). A small fraction of the electrons that pass through complex 1 and complex 3 react with oxygen to form the superoxide anion [38]. Complex 1 releases the superoxide anion into the mitochondrial matrix, while complex 3 releases superoxide



**Fig. 11** Formation and inactivation of reactive oxygen species (ROS) in mitochondria. Most of the electrons that enter the respiratory chain finish in cytochrome *c* oxidase (complex 4), where four electrons are added quickly and in a tight cage to oxygen, so that ROS are not released, but only water is safely formed. However, upstream to complex 4, a few of the electrons donated to the respiratory chain react with oxygen to form the superoxide anion radical ( $O_2^-$ ). Complex 1 generates  $O_2^-$  on the matrix side. Complex 3 forms  $O_2^-$  both in the matrix and in the intermembrane space. In the matrix, manganese superoxide dismutase (MnSOD) dismutates two molecules of the superoxide anion into one oxygen molecule and one molecule of hydrogen peroxide ( $H_2O_2$ ). Glutathione peroxidase 1 (GPx1) then reduces  $H_2O_2$  into water while oxidizing two reduced glutathione (GSH) molecules into glutathione disulphide (GSSG). Glutathione reductase (GR) then regenerates GSH at the expense of NADPH. Finally, an energy-linked NAD(P)<sup>+</sup> transhydrogenase (TH) uses NADH and the mitochondrial membrane potential to regenerate NADPH from NADP<sup>+</sup>. In the intermembrane space, the superoxide anion is detoxified by its reaction with oxidized (ferric) cytochrome *c* to form reduced (ferrous) cytochrome *c*, which donates its electron to complex 4 of the respiratory chain, thus regenerating oxidized cytochrome *c*. Although copper zinc superoxide dismutase (CuZnSOD) is mostly cytosolic, it is also imported into the intermembrane space of mitochondria, where it dismutates  $O_2^-$  into  $H_2O_2$ . Although most of the ROS formed by mitochondria are detoxified within mitochondria, some molecules of  $H_2O_2$  can cross the outer membrane. In the acidic intermembrane space,  $O_2^-$  may be protonated to the uncharged, hydroperoxyl radical ( $HO_2^-$ ), which may also cross the outer membrane to regenerate  $O_2^-$  in the cytosol. IM, inner membrane; OM, outer membrane.

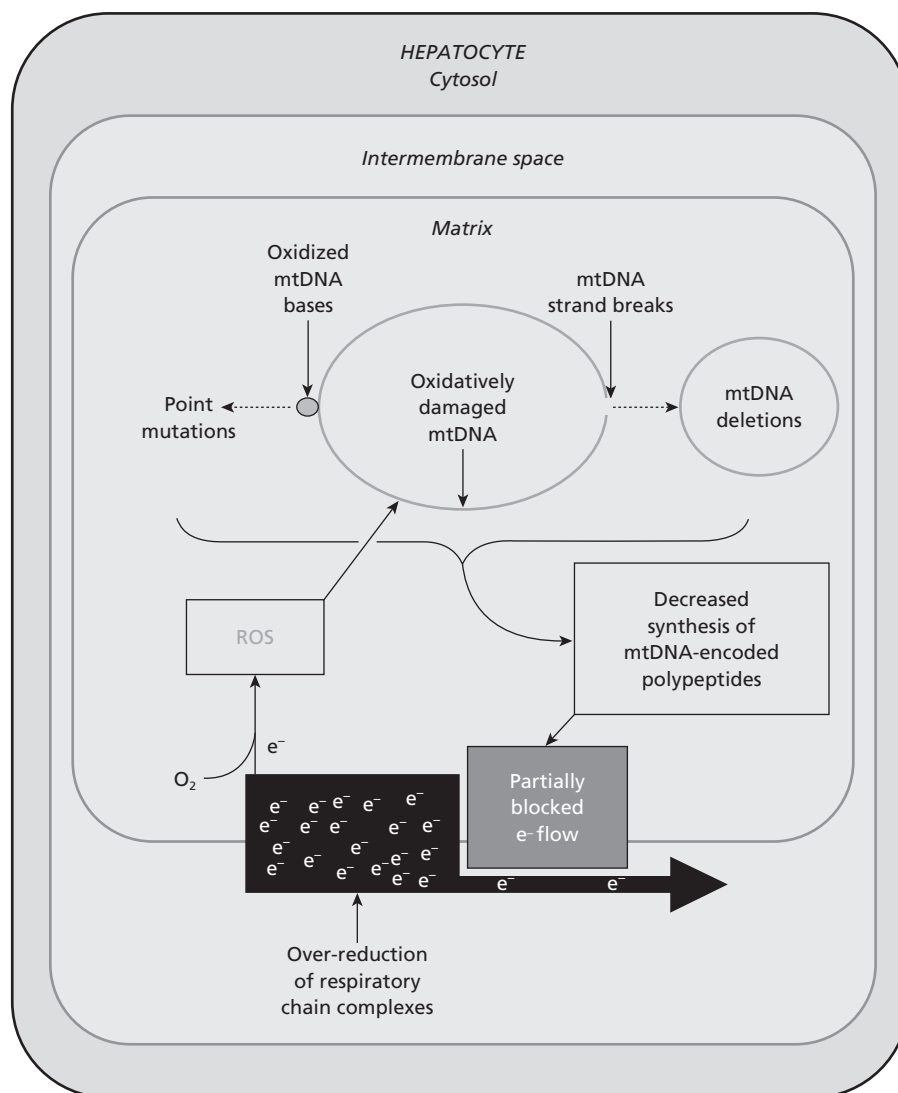
into both the mitochondrial matrix and the intermembrane space (Fig. 11) [38]. Other possible site(s) for superoxide formation may be ETF (electron transfer protein) and/or ETF:QO (ETF:quinone oxidoreductase) [38]. Superoxide generation by ETF and/or ETF:QO has been proposed as one possible explanation for the higher formation of superoxide when muscle mitochondria are energized with palmitoyl carnitine than with complex 1 substrates [38]. Overall, it is estimated that 1–2% of the oxygen consumed by mitochondria may be transformed into the superoxide anion, and that mitochondria represent the most important source of ROS in cells [39].

Being unable to completely prevent ROS formation, mitochondria have devised several systems to eliminate these ROS (Fig. 11). In particular, the superoxide radical anion is dismutated by mitochondrial manganese superoxide dismutase into hydrogen peroxide, which is detoxified into water by mitochondrial glutathione peroxidase 1 (Fig. 11) [39].

Nevertheless, residual levels of ROS can impair mitochondrial structure and function. The superoxide anion can remove one iron molecule from the cubane ( $4\text{Fe}-4\text{S}$ ) cluster of mitochondrial aconitase, thus inactivating aconitase and hampering fuel oxidation in the tricarboxylic acid cycle [40]. ROS can also damage mitochondrial proteins, cardiolipin and mtDNA, thus progressively impairing mitochondrial function [41]. In some pathological conditions, a very large formation of mitochondrial ROS can trigger, or can help to trigger, mitochondrial permeability transition, which irreversibly disables mitochondria and leads to their autophagic degradation [42].

Finally, some molecules of hydrogen peroxide and possibly also of the superoxide anion (the latter probably crossing the membrane as the hydroperoxyl radical) may leak out of mitochondria [43,44] and may eventually damage extramitochondrial cell constituents, such as nuclear DNA. However, mitochondrial constituents, including mtDNA, are the main targets of mitochondrial ROS.

**Fig. 12** Ageing of mtDNA. Reactive oxygen species (ROS) formed by the mitochondrial respiratory chain damage mtDNA. They form oxidized mtDNA bases, which may occasionally cause point mutations, and they also form mtDNA strand breaks, which may occasionally cause mtDNA deletions. Eventually, these mtDNA lesions and mutations may decrease the synthesis of mtDNA-encoded polypeptides, thus partially hampering the flow of electrons in the respiratory chain. In the overly reduced complex 1 and complex 3, the accumulated electrons may increasingly react with oxygen to form the superoxide anion radical. The increased ROS formation may further damage mtDNA. This vicious cycle, together with a decreased import of DNA repair enzymes as a consequence of the decreased membrane potential, could explain why mtDNA deletions and point mutations, which are exceptional before 40 years of age, then start to accumulate exponentially during old age.



## Ageing of mitochondrial DNA

Mitochondrial ROS play an important role in the ageing process (Fig. 12). ROS oxidize mtDNA bases to form, in particular, 8-oxo-deoxyguanosine. During mtDNA replication, this modified guanine can mispair with an adenine (instead of a cytosine), and this mispairing can cause mtDNA point mutations. ROS also cause mtDNA strand breaks, which can occasionally lead to mtDNA deletions.

As these mutations accumulate over the years, they may eventually decrease the synthesis of mtDNA-encoded polypeptides, thus partially blocking the flow of electrons in the respiratory chain (Fig. 12). The over-reduction of upstream respiratory complexes may then increase mitochondrial ROS formation. Furthermore, as the mitochondrial membrane potential decreases, the membrane potential-driven import of DNA repair enzymes from the cytosol into the mitochondrial matrix may become impaired [45]. Thus, both increased mtDNA damage (due to the increased mitochondrial ROS formation) and decreased mtDNA repair (due to decreased import of repair enzymes) could explain why mtDNA deletions and point mutations increase exponentially during old age [46,47].

## Overview

When photosynthesizing cyanobacteria began releasing oxygen in the atmosphere, bacteria took advantage of their quick turnover, and the high mutation rate caused by oxygen-derived ROS, to mutate and adapt to the oxygen environment. They developed truly wonderful biochemical machines, enabling them first to survive and then to thrive with energy in the new oxygen environment. Instead of trying to emulate the biochemical perfection of bacteria, other forms of life have associated with them. Assisted and controlled by nuclear genes, our mitochondrial partners oxidize or save fuels as needed, just to provide cells with the exact amount of energy they need.

However, as in all partnerships, there is some hardship. We are totally dependent on mitochondria, so that their failure is also a significant threat to health. Inborn defects, drugs or cytokines that impair mitochondrial function can cause steatosis, cell dysfunction or cell death. Furthermore, although mitochondria provide energy, they also form reactive oxygen species that contribute to ageing and death.

Death and reproduction have permitted the slow selection and adaptation of the host, providing mitochondria with an ever-adapting environment. While new species of the host have arisen and then become extinct, the mitochondrial clones have endured.

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## 2.3.5 Bilirubin metabolism

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### Bilirubin in medical history

Bilirubin has attracted the attention of physicians since antiquity. Its chemistry, metabolism and disposal have been studied systematically during the last two centuries as a model for hepatic disposal of biologically important organic anions of limited aqueous solubility [1]. The discovery of several inherited disorders of bilirubin metabolism and excretion during the twentieth century has led to renewed interest in inherited diseases associated with jaundice, some of which continue to pose a therapeutic challenge, providing impetus for further research. While physicians are mainly concerned with the toxic effect of bilirubin and its importance as a liver function test, the antioxidant property of bilirubin may provide a physiological defence against oxidative injury.

### Formation of bilirubin

#### Sources of bilirubin

Bilirubin is the breakdown product of the haem moiety of haemoglobin, other haemoproteins, such as cytochromes, catalase, peroxidase and tryptophan pyrrolase, and a small pool of free haem. In humans, 250–400 mg of bilirubin is produced daily, of which approximately 20% is produced from non-haemoglobin sources [2]. Following the injection of radio-labelled porphyrin precursors (glycine or  $\delta$ -aminolaevulinic acid), an ‘early-labelled peak’ of bilirubin (ELP) is excreted in bile within 72 h [3]. The initial component of ELP is derived mainly from hepatic haemoproteins. This is followed by a slower component, derived from both erythroid and non-erythroid sources, which becomes prominent in conditions associated with ‘ineffective erythropoiesis’, e.g. congenital dyserythropoietic anaemias, megaloblastic anaemias, iron-deficiency anaemia, erythropoietic porphyria and lead poisoning [4], and in accelerated erythropoiesis [5]. The ‘late-labelled peak’ appears at approximately 110 days in humans and coincides with the half-life of erythrocytes.

#### Enzymatic mechanism of bilirubin formation

The microsomal haem oxygenase (HO) enzymes catalyse the oxidation of haem (Fig. 1). Three molecules of  $\text{O}_2$  are consumed