Hypothesis

Does F_1-ATPase subunit γ turn in the wrong direction?

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Abstract Analyzing the direction of F_1-ATPase subunit γ rotation, its shape and non-random distribution of surface residues, a mechanism is proposed for how γ induces the closing/opening of the catalytic sites at β/α interfaces: by keeping contact with the mobile domain of subunits β at the 'jaw' (D386, the seven consecutive hydrophobic residues and D394/E395), rotating γ works as a screw conveyer within the barrel of $(\alpha,\beta)_3$. Mutations of the conveyer contacts are predicted to inhibit. Rotating wheel cartoons illustrate enzyme turnover and conformational changes. Steric clashes, polar interactions and also substrate limitations lead to specific stops. Because it is constructed as a stepper, γ prevents uncoupling at high energy charge.

Key words: ATP synthesis; Rotation; Coupling; Hydrogen bond

1. Direction of γ rotation discrepancy

1.1. Occupancies versus conformational states

ATP is synthesized by F_1-ATPases in chloroplasts, mitochondria and eubacteria in high affinity tight catalytic sites, probably at a low H_2O potential [1]. The required free energy is used to bind inorganic phosphate (P_i) and to release ATP [2–13]. It is relayed indirectly, i.e. mechanically, to the three active sites by a rotation of subunits γ/ε against $(\alpha,\beta)_3$. This rotation, in turn, is energized by a rotation of the connected membrane integral rotor of F_oc subunits driven by H^+ efflux. A topological problem arises, however, when the information on (1) the *direction* of the rotation of subunits γ/ε in the bacterial enzyme(s) during ATP hydrolysis [14,15] and (2) the X-ray structure of the bovine enzyme [16–18] is correlated with (3) the sequence of kinetic conformational states [2–9].

In the X-ray structure of bovine F_1, different sectors of the asymmetric subunit γ are in contact with the three subunits β in different conformations [16]. The relative position of the occupancies **E** (empty), **TP** (triphosphate binding) and **DP** (diphosphate binding) of the three β subunits is counter-clockwise, when viewed from above. Therefore, if the interactions with subunit γ determine the conformation and occupancy of the three different subunits β, the direction of rotation of subunit γ driven by ATP hydrolysis could have been deduced to be clockwise relative to the fixed catalytic sites, viewed from above. Accordingly, in videograms of ATP hydrolysis-driven movements of devices fixed to subunit γ the direction of rotation is clockwise, viewed from above, because it is counter-clockwise, when viewed from the membrane side [14,15,19].

Mainly from exchange rates in pulse/chase experiments, on the other hand, three conformational states have been defined, **O**pen with low, **L**oose with medium and **T**ight with high affinity to nucleotides, and the 'energy-linked binding change mechanism' has been formulated [2–7]. In the assignment [16] of the occupancies to these conformational states, E corresponds to O, TP to L and DP to T. This assignment suggests that the sequence of conformational changes during ATP hydrolysis is O-L-T-O. From kinetic analyses this O-L-T-O sequence has been deduced for ATP *synthesis* [2–10,20], however.

The assumption that in the videograms γ turns artificially in the wrong way would lead in reality to the sequence O-T-L-O in ATP hydrolysis, in agreement with the deduced sequence of the three kinetic conformational states; but this consideration would also require the interchange of the occupancies β_{DP} and β_{TP} in the X-ray structures. Interchanging the assignment [21] or introducing the additional conformation 'closed' into the static three-sector cartoons [10] does not solve the problem either. The 'direction of rotation discrepancy' [22] is represented in this article as two three-step cartoons (Fig. 1a,b), showing the course of events in one catalytic site correlated to the three conformational affinities. The data and conclusions represented in Fig. 1a include the loose conformational state L during ADP binding (and release), whereas the conflicting data and conclusions represented in Fig. 1b include the loose conformational state L during ATP release (and binding).

It has been deduced [11,12] that ATP binding, hydrolysis and product release do not occur at stop positions (seen in the X-ray structure) but *during* rotation of γ, drawn by us as a multistep cartoon in Fig. 1c. The proposed ATP 'binding zipper' [11,12] describes a multistep binding of ATP onto the active site before ATP hydrolysis, causing the conformational change $\beta_E \rightarrow \beta_{TP}$ and forcing subunit β to push γ into a clockwise rotation, the first power stroke [11,12]. In reverse,

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Abbreviations: MEP, most eccentric point; P_i, inorganic phosphate anion; Amino acids are mostly given in the single letter code, e.g. γM23K means a mutation of residue subunit γ methionine 23 to lysine

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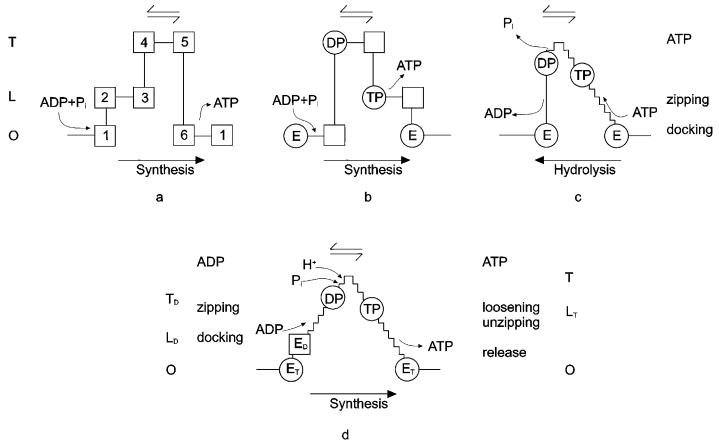

Fig. 1. Correlation of conformational changes with the sequence of events in one catalytic site of F_1-ATPase. Schematic step representation of the three-sector cartoons of (a) Boyer [2], Duncan et al. [9] and Pänke et al. [20]; (b) Abrahams et al. [16] and Boyer [3]; (c) the proposal by Oster and Wang [12]; (d) the new multistep hypothesis for both ADP binding and ATP release. The *x*-axis (time of events) and *y*-axis (affinity of conformations) are schematic and not to scale. T, L, O = tight, loose and open [2–5], i.e. high, medium and low [6,7], affinities of the binding sites; L_D, L_T, T_D = loose binding of ADP, ATP and tight binding of ADP, respectively. E_T = empty after ATP release; E_D = empty before ADP docking. Squares: kinetic conformational states; 1, open empty; 2, loose empty (abandoned later); 3, loose with ADP and P_i; 4, transient tight with ADP and P_i; 5, tight with ATP; 6, open for ATP release. Circles: occupancies in X-ray structures [16,17]; ⇋ = catalysis.

this would mean an energy-dependent multistep ATP release after ATP synthesis; β_{TP} does not represent the tight transition state (T), but already a partially loose ATP binding (L_T).

If, as in this article, the concept that major catalytic events occur during rotation of γ is extended to ATP synthesis, and if besides two tight states two different loose states are proposed, at least one before and one after catalysis (Fig. 1d), the three approaches can be combined without leading to a discrepancy. (Mg·ADP and Mg·ATP are the true substrate and product, respectively [2–7,16–18].)

1.2. Tight ADP binding and loose ATP binding

From the pdb files 1bmf and 1e1q from bovine F_1 it was suggested that β_{DP} binds ADP tightly, and that β_{TP} is structurally more loose, since α_{DP}R373 is in contact with the substrate but α_{TP} R373 has moved [16]. As can be seen more clearly from pdb file 1e79 [17], β_{DP}F424 and Y345 stagger with the adenosine moiety, indeed, whereas β_{TP}F424 is tilted and mobile (higher B-factor). In addition the conical access tunnel [16] in the catalytic β/α interface is closed to less than 2 Å in DP, both to the medium and to the internal cavity, whereas in TP it is as open as in E; this was seen when an internal cavity was generated from pdb file 1e79 or 1e1r with the program SURFNET [23] (data not shown). The number of contacts at the catalytic interfaces in 1bmf is 93 in E, 176 in DP and 131 in TP [24]. Thus the assignment [16] that the structurally tight and nearly inaccessible β_{DP} reflects a high affinity conformational state (T_D) and that the structurally less ordered and well accessible β_{TP} reflects a loose state (L_T) seems inevitable. (Note: All measurements and conclusions – cited or drawn by us – rely on diffraction data with no better than 2.0 Å resolution.)

1.3. A rotating wheel cartoon, showing two different loose and two tight states

Subunit γ rotates clockwise, viewed from above, driven by ATP hydrolysis [14,15,19], and is expected to rotate counterclockwise driven by the torque produced by ΔpH on the connected F_oc ring. The observed direction of rotation is in accordance with the sequence: substrate uptake, catalysis and product release in both directions. If these tasks are performed during rotation of γ, i.e. *between* stop structures of β, the X-ray occupancies β_E, β_{DP} and β_{TP}, one does not see the structure of the enzyme during catalysis, i.e. the tight kinetic conformational 'state' (T), in any pdb file before publication of 1h8e [18]. In crystallized 'native' F_1 no loose ADP binding has been seen [16,17], and in the diverse kinetic analyses no loose state after ATP synthesis has been needed to interpret the data [9,20].

The conformational change that induces ADP binding is brought about by γ rotation. We propose to consider a multistep ADP binding during the conformational change $\beta_E \rightarrow \beta_{DP}$, and in reverse a multistep ADP release after hydrolysis, including loose ADP binding structure(s) (L_D). Since the way the different enzymatic events and the changes of conformational states have been illustrated in three-sector cartoons so far is confusing, we have developed a new kind of representation, the 'rotating wheel cartoon' (Fig. 2a,b). The wheel cartoon is not supposed to be an animation of the γ rotation, but to define which events at the β/α interfaces are simultaneous with and/or induced by the γ rotation, and to illustrate the endless repetitive sequence of the concerted conformational changes in the transposed rhythm of a trisite mechanism. Two different non-tight conformations during ADP binding and ATP release (L_D, L_T), two different tight conformations, the tight ADP binding T_D and the tight transition state T, and an empty (= open) state are included. Naturally not more than three different structures can be found in any crystallized trimeric F_1.

In the recent X-ray structure of beef heart F_1, pdb file 1h8e, subunit γ is said to be stopped about 30° counter-clockwise [18]. Former β_{TP} is still in the process of zipping in ATP, former β_{DP} is concluded to be shown in the transition state (T) and former β_E in a pre-product release state [18]. The half-closed conformation between β_E and β_{DP} shows a loosely bound ADP and a transient SO_4; residues βY345 and F424 are not yet tightly staggered with the adenosine moiety in $\beta_{E\ half\ closed}$ as compared to β_{DP}, and αR373 has not yet closed

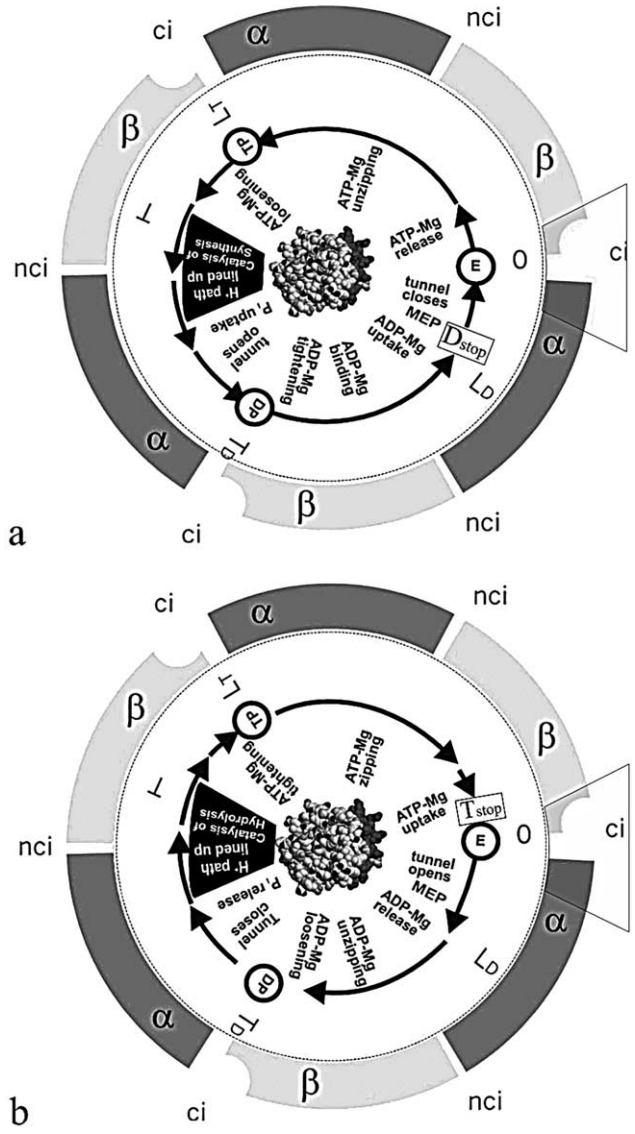

Fig. 2. Rotating wheel representation of the sequence of events at the catalytic interface (on the right side) of F_1-ATPase, viewed from above, (a) ATP synthesis, (b) ATP hydrolysis. The reader is asked to copy the figure, cut out the inner circle along the dotted line and turn the wheel within the outer $(\alpha,\beta)_3$ barrel. Abbreviations as in Fig. 1. Arrows in a: counter-clockwise rotation of γ, viewed from above, determining the β conformational changes during ATP synthesis; arrows in b: clockwise rotation of γ, viewed from above, during ATP hydrolysis. Square in a: 'state 4 stop' due to low ADP; square in b: 'video stop' due to ATP deficiency. MEP = most eccentric point [11,12]. ci, catalytic interface; nci, non-catalytic interface. For further explanations see text.

in completely. The three β structures in the former pdb files, the 'native' structure [17], are easily combined in the rotating wheel cartoon with the three new structures, Fig. 2. Thus the sequence of conformational states in our proposal would be O-L_D-T_D-T-L_T-O for ATP synthesis (Fig. 2a). In their recent review Senior et al. [8] deduce a hypothetical trisite mechanism of ATP synthesis requiring six different conformational states [8]. The three-sector cartoons showing only three conformational states, which are frequently used also in textbooks, are due to be replaced.

The rotating wheel cartoon illustrates that – except for the transient β_E – all three sites are occupied by nucleotides [2–8]; ATP binding ($\beta_E \rightarrow \beta_{TP}$) leads to a clockwise rotation of γ, viewed from above [14,15,19] (Fig. 2b), whereas the counter-clockwise rotation of γ leads to ADP binding ($\beta_E \rightarrow \beta_{DP}$) (Fig. 2a). The rotating wheel cartoon even offers the possibility to illustrate the time needed for a certain event at the β/α interfaces by allotting different widths to the sectors on the wheel describing the respective events.

The problem, however, why not more than three conformational states have been needed to calculate kinetic data from exchange experiments [2–10,20] (Fig. 1a,b), still remains puzzling. Senior et al. [8] argue that exchange experiments were done at extreme substrate limitation and therefore results and conclusions would not be applicable to the enzyme working at physiological conditions.

2. How are γ rotation and β movements interlocked?

2.1. Non-random distribution of residue types at the surface of γ

For crystallization of isolated F_1 movements have been stopped and subunits have been fixed by various inhibitors of activity. Analysis of the structures have so far revealed very similar relative positions of the highly asymmetric γ inside the $(\alpha,\beta)_3$ barrel. In Table 1 we list all polar interactions of γ with the large subunits found in bovine F_1 (stop positions in pdb files 1e79, 1e1q and the recent 1h8e) up to 3.5 Å. Some of the 'catches' had not been mentioned explicitly [16–18]. The distances of the respective H donor and acceptor atoms are listed; the directionality is not analyzed.

The N-terminal residues 1–54 of subunit γ form an antiparallel left-handed coiled coil with C-terminal residues, kept in place by the upper hydrophobic sleeve and extending through the internal cavity of the $(\alpha,\beta)_3$ barrel [16]. The major part of γ building a Rossman fold-like structure is densely packed onto this coil and constitutes most of the exposed central stalk not in contact with α or β [17]. We recognized a strikingly high proportion of positively charged residues among the N-terminal residues, and in addition – analyzing the structure in 'spacefill' instead of in the 'ribbon' mode – a significant non-random distribution of surface residues. On the 'ADP side' of γ (stop positions $\beta_{E\rightarrow DP}$), there is a continuous left-handed ridge of positively charged residues (K24, K21, K18, N15, K11, R8), leading upwards, and a continuous left-handed collar (R36, R33, K30/R134, R133, R75+K111) below. These two rails enclose a groove with no charges and mostly hydrophobic residues (Fig. 3). Between the catches to β_{DP} and β_{TP} the upper ridge on γ continues in R8, R9, N238, while the lower collar continues in R75+K111, H82, K87/K90 (structure not shown). On the 'ATP side' ($\beta_{TP\rightarrow E}$) there is a right-handed slope of polar residues on γ (N238, N234, E241, K237, D233, K24), a right-handed discontinuous positively charged collar (K90/K87, Y171/T173, K172, R166/R164, R42, K39, R36) and a non-polar groove in between (structure not shown).

2.2. Conveyer action of γ

If in F_1-ATPases the hydrolysis of three ATP/one 360° revolution generates the torque in three large steps, an elastic power transmission is needed, since the torque output from $F_1\gamma$ is measured to be smooth and continuous. The enzyme therefore can cope with the symmetry mismatch of three cat-

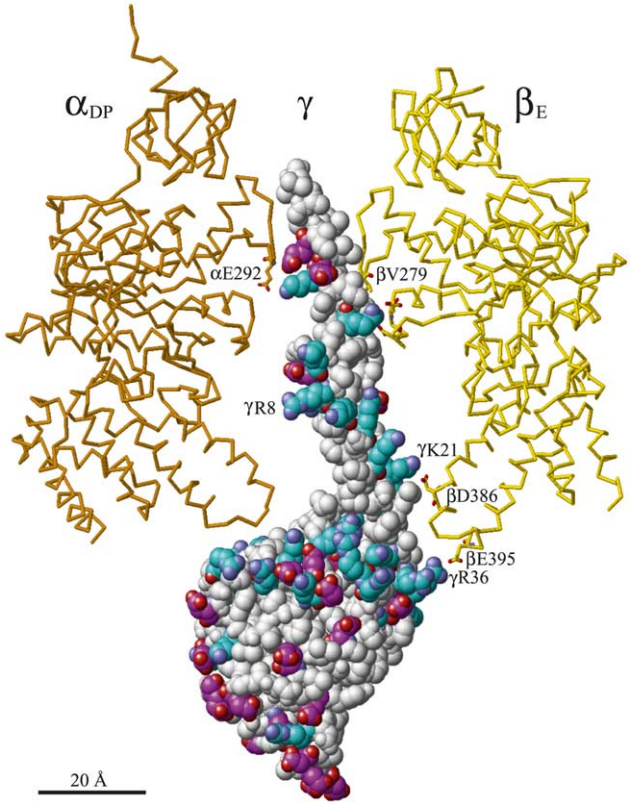

Fig. 3. Shape and non-random distribution of residues on the surface of the 'conveyer' F_1 subunit γ. Cross-section of bovine F_1, ADP sector (E→DP) of γ in front, produced from pdb file 1e79 by RasMol simulation [45]. Subunits α_{DP} (orange) and β_E (yellow) shown in backbone; residues of polar interactions in sticks; γ (white) in spacefill, acidic residues in magenta, O in red, basic residues in cyan, N in blue. Scale bar = 20 Å.

alytic interfaces in F_1 and 10–14 H^+-transporting F_oc subunits [25]. Still subunit $F_1\gamma$ is regarded to be rather stiff [11,12,17,26,13]. We propose that it works *like a screw conveyer* due to its very structure and the described distribution of surface residues: in the conformation β_E, βD386 is close to γK24, βE395 is in contact with γR36 and the seven hydrophobic residues in between, especially I390/L391, are in contact with the hydrophobic groove on γ (Fig. 3). Due to counter-clockwise rotation of γ the described particular structure on γ guides the C-terminal helical domain of β smoothly upwards in small steps during ADP binding, βD386 running along the upper ridge, βD394/E395 along the collar below, and the seven hydrophobic residues gliding through the hydrophobic groove. This motion of helices 1 and 2 of the C-terminal helical domain and the loop in between (a helix-turn-helix motif called the jaw [21]) is connected with the hinge-bending motion [11,12] of helices B and C and with changes in H bonds between strands 3 and 7 [16] that close the active site.

When the γ 'catalytic plateau' ($\beta_{DP\rightarrow TP}$) rotates counter-clockwise underneath the β jaw, β stays lifted up. The catalytic catches of β_{DP} [17] change to the H bond γN238ND–β_{TP}A389O/β_{TP}I390O (Table 1). Due to counter-clockwise rotation of γ the described right-handed structure on the 'ATP side' of γ would force the β jaw down and outwards, to unzip and to release ATP.

Specific electrostatic and hydrophobic interactions between residues on subunits γ and the 'jaw' of β thus contribute to dictate the structure of β; the rotation of subunit γ, being geared with up and down motions of the mobile domain of β, forces and/or allows specific sequential conformational changes. The individual contributions and interaction forces of the peculiar track-groove-track pattern around subunit γ with the $(\alpha,\beta)_3$ barrel still need to be analyzed. The distances of residues βD386 and βE395 on average match with the distance of the upper and lower positively charged ribbons. We speculate that the seven hydrophobic residues of β also keep contact with the hydrophobic groove on γ. In a recent biased molecular dynamic simulation some of the energy transfer events between subunit γ and the $(\alpha,\beta)_3$ barrel during ATP synthesis were investigated; γN238, R36 and M25 are identified as hot spots [13] (see below).

2.3. Accessibilities during substrate uptake and product release

The following partially hypothetical sequence of events during ATP synthesis can be deduced (Fig. 2a). After ATP release the site is accessible but empty (E), since the affinity is low; β_EE395 is bonded to γR36 (1e79) and the conical access tunnel [16] is open. Due to energization of the respective membrane H^+ efflux turns γ via the F_o assembly counter-clockwise (viewed from above) by about 30° [18], overcoming the 'most eccentric point' (MEP) (see below); β_EE395/D394 is moved to γR33 as seen in the AlF_4-inhibited structure 1h8e (Table 1). The six polar interactions of the 'catalytic catch' [17] of γ with β_{DP}/α_E do not all disrupt immediately (γR75 keeps bonded to βE395, Table 1), but shift the entire dimer β_{DP}/α_E counter-clockwise sidewards, thus closing the conical tunnel on former β_E [18] (and opening the tunnel on former β_{DP}!). The site on β_E gains medium affinity (L_D) for ADP, which enters e.g. from underneath. Upon further rotation of γ by about 90° the transiently seen SO_4 (PO_4) [18] is removed, the site is closed in multiple steps and ADP zipped into the tight binding (T_D) found in β_{DP} [16,17]. Then the tunnel would open (see above) and P_i enter, attracted by βR189/βR191. Residues βK162 and αR373 are already engaged in binding the α- and β-phosphoryl of ADP.

When the γ 'catalytic plateau' rotates further underneath the β jaw (the black box in our rotating wheel cartoon), both D386 and D_{394}ELSEED are not H-bonded to positive charges on γ for a short time. A composite proton path (part of Mitchell's rolling proton well [27]?) may form intermittently to draw the chemical H^+ from the bulk to the active site (residues suitable to participate in this hypothetical path: βE398, βD394, γH82, γC78; βE395, γM232, γT20, γS12, βD386, γD5, βD315, βD316, βY311, βE188).

Catalysis in a transient tight conformational state (T) seems to occur after βD386 is bonded to γR9 (Table 1). Since γR75 is still bonded to βE395, but γK111 not any more to βD394 [18], the β jaw is tilted as compared to former β_{DP} when γ dwells about 30° counter-clockwise. Chemical details of catalysis are not within the scope of this article focussing on the peculiarities of the structure; they remain to be identified and agreed upon as discussed elsewhere [2–8,17,18,27,28]. The nucleotide is considered to be tightly bound [1] but the site is not closed (i.e. inaccessible to H_2O), since ^{18}O still exchanges between bound P_i and H_2O in an uncoupler-insensitive reaction several times before ATP release [2–5]. A larger cavity with secluded H_2O described earlier in chloroplast CF_1 [29] was not found in the analysis of bovine F_1.

Table 1
Distances between residues of MF_1 subunits γ and α/β forming 'catches', i.e. H-bonded ion pairs or putative H bonds up to 3.5 Å in bold, and the longer non-bonding distances between the same residues in different γ stop positions, respectively (taken from pdb 1e1q, 1e79 and 1h8e)[a]

γ		α/β	Å 1e1q	Å 1e79	Å 1h8e
		β_E (E)			
T259 OG[b]		V279 N	**2.98**	**2.79**	**2.67**
R254 NH_1		D316 OD_1	**3.14**	**3.10**	**2.75**
		D316 OD_2	**3.22**	5.16	4.68
		D319 OD	**3.40**	**3.02**	**3.16**
Q255 NE		D316 OD_1 [b]	**2.87**	**2.74**	**2.73**
		T318 OG	**2.81**	**3.08**	**3.10**
R36 NH_1 [b]		E395 OE_1	9.00	**3.11**	6.32
R36 NH_2 [b]			8.89	**3.14**	6.64
R33 NH[b]		E395 OE	13.29	9.56	**3.13**
		α_E (A)			
K30 NZ[b]		D409 OD	**2.92**	3.76	?[c]
R75 NH_1	c[e]	D409 OD	?[d]	**3.00**	?[c]
K18 NZ[b]		E399 O	4.27	3.73	**2.63**
		β_{DP} (D)			
E261 OE[b]		V279 N	4.19	4.14	**3.04**
R8 NH[b]	c	D386 OD_1	**3.25**	**3.37**	12.00
		D386 OD_2	**3.45**	4.16	10.18
R8 NE[b]	c	D386 OD_2	**2.98**	**3.38**	12.23
R9 NH[b]		D386 OD_1	10.98	10.09	**2.70**
		D386 OD_2	11.44	9.58	**3.04**
K111 NZ[b]	c	D394 OD	?[d]	**2.81**	5.41
R75 NH_2	c	E395 OE_1	?[d]	**3.15**	**3.33**
R75 NE	c	E395 OE_2	?[d]	**3.19**	**3.16**
		α_{DP} (C)			
E264 OE[b]		E292 N	3.58	**2.70**	**2.50**
		β_{TP} (F)			
N238 ND[b]		A389 O	**3.30**	**2.77**	**3.16**
		I390 O	**2.91**	**3.37**	**2.94**
K87 NZ		E398 OE_1	**2.70**	5.11	5.44
		E398 OE_2	**3.18**	7.21	6.89
		α_{TP} (B)			
R252 NE[b]		D333 OD	**3.32**	3.56	3.80
R252 NH[b]		D333 OD	4.58	5.46	**3.41**

[a]Distances are measured with RasMol and taken from the atomic models that were calculated from the X-ray diffraction data with 2.6, 2.4 and 2.0 Å resolution, respectively.
[b]Described for the first time.
[c]α_E 404–409 not resolved in pdb file 1h8e.
[d]Not resolved in pdb files 1e1q and 1bmf.
[e]c, 'catalytic catch' in pdb file 1e79.

In the stop position β_{TP} an H_2O molecule is seen on βE188, suitable to have been built in catalysis [16]. The ATP formed is already loosened and partially unzipped in β_{TP} with γN238 H-bonded to the β jaw (L_T). Upon further counter-clockwise rotation of γ, βD386 will collide with γE241 and again with γD233 and force the jaw into sudden downward motions to open the site completely and to unzip and release ATP along a different route into the bulk (stroma).

In this speculation ADP uptake and ATP release could be guided by surplus positive charges on γ, and γ could conduct the chemical H^+ along a transient composite proton path. This means γ would guide a negatively charged substrate to the active site along positive charges at its surface, and afterwards the H^+ to the same site along negative charges, facilitated by rotation.

The deduced sequential non-random binding of ADP and P_i (0.2–0.4 mM and 4–15 mM in illuminated chloroplasts, respectively [30]) could make sense in ATP synthesis under physiological conditions. A competition of tight ADP binding (zipping) by excess P_i would be prevented by closure of the conical access tunnel after ADP docking. Although P_i binding before ADP binding is not excluded under conditions of unisite catalysis, i.e. severe substrate limitation [3,20,28], from other data it has been concluded that only ADP binding creates a P_i binding pocket [6–8]. In reverse, after ATP hydrolysis, a sequential release of products has been correlated with the proposed second power stroke [11,12], and with the additional dwell at 90° of clockwise γ rotation (viewed from above) [15].

3. Artificial and functional stops in γ rotation

3.1. Definition of different kinds of stops

F_1 subunit γ transduces energy. Although it is not part of the internal surface of the catalytic site [16–18], γ controls its activity. It induces and coordinates the conformational changes of subunits β. A specific structural basis of this coordination is proposed: βD386 and D394/E395 of D_{394}ELSEED keep contact with the positively charged ribbons on γ; existing catches must disrupt and new catches are formed permanently in small steps, whereas the seven hydrophobic residues glide through the non-polar groove, especially βI390/βL391. The distance of βI390 in the jaw to βK162 in the P loop of the active site is between 33 and 37 Å in all known conformations. The energetic consequence of a more general 'β crawling on γ' has been discussed [31].

During ATP synthesis the speed of step rotation of γ limits the catalytic turnover on β, and during ATP hydrolysis the conformational changes of β drive and limit the rotation of γ. At least three kinds of stops in γ rotation should be considered: an 'X-ray stop' must have been obtained before crystallization, in 1e1q by the substrate-analogue adenosine 5′-(β,γ-imino)triphosphate, in 1e79 by the ATPase inhibitor *N*,*N*′-dicyclohexyl carbodiimide, reacting with β_{DP} E199 [17], and in 1h8e by the PO_4 analogue AlF_4 [18]. In the video analysis of the γ rotation in individual F_1 molecules [14,15,19] driven by ATP hydrolysis, it was seen that the rotation occurs in 120° steps, but only if ATP was limiting [14]; with a different experimental device a much better time resolution was achieved and an additional dwell at 90° (clockwise, viewed from above) was observed [15]. If one assumes that the 'video stop' in γ rotation due to ATP deficiency occurs in the same position relative to α/β as the X-ray stop due to inhibition, the sequences of events result that are shown in the rotating wheel cartoons (Fig. 2).

In addition we propose to consider a physiological 'state 4 stop' in counter-clockwise γ rotation during ATP synthesis due to low concentration of ADP, i.e. high energy charge, and discuss its significance.

3.2. Significance of stops in γ rotation

Hydrophobic interactions alone are not specific enough to fix γ in a certain stop position. The crystal structures of bovine F_1 (pdb files 1e1q and 1e79) suggest, indeed, that the 120° stops in γ rotation are due to 'catches', i.e. specific polar interactions between γ and the $(\alpha,\beta)_3$ barrel. This conclusion is supported by the findings that AlF_3 (pdb file 1e1r), and the ATPase inhibitors aurovertin (pdb file 1cow) and efrapeptin (pdb file 1efr) cause a very similar X-ray stop structure, i.e. essentially the same structure within the limits of resolution. Even the crystallized α,β,γ trimer from rat liver mitochondrial F_1 [32] (before generating the multimeric structure by BIOMT transformations and omitting overlapping γ two times (pdb file 1mab)) shows a very similar stop structure: residue γN235 (corresponding to bovine N238) is in 2.8 Å contact with βI390 and γE261(bovine 264)OE is seen 3.6 Å from αE292N, thus identifying the crystallized large subunit pair as β_{TP} and α_{DP} (cf. Table 1).

But in file 1h8e, claimed to show a different γ stop position, in part about 30° counter-clockwise [18], only three out of 12 possible polar interactions are different (Table 1). Therefore, in addition, steric clashes may cause the specific stop structures, and the catches correspond to the resulting relative positions.

No asymmetric F_1 structure has been described so far with a closed but empty catalytic binding site. According to current hypotheses no rotation of γ occurs without movements within $(\alpha,\beta)_3$ and vice versa. Since structural and energetic barriers have to be overcome [6–8,13,25,46] no rotation is expected without energy input. The 'video stops' in clockwise γ rotation due to ATP deficiency suggest that binding of each ATP molecule drives one β_E to close and to push γ into one 120° rotation step [11,12,14,15]; hydrolysis is concluded to induce product release to allow repetition of the cycle [11,12]. The recent first molecular dynamic simulation of movements within F_1 during ATP synthesis (unfortunately starting with pdb file 1bmf with incomplete γ, missing e.g. γR75, K111 and H82) showed that isolated solvated β_E closed fast and spontaneously, triggered by counter-clockwise γ rotation to remove steric obstructions, but without nucleotide binding [13]. Therefore opening, in reverse, and ADP release after ATP hydrolysis would be an energy-consuming step, in contrast to most conclusions drawn so far [2–8,11,12]. The crystal structure of the thermophile TF_1, without bound nucleotide and without subunit γ, also shows open β_E only (pdb file 1sky) [33]. Newly synthesized ATP is not released without ADP binding to the next sites, even in the presence of excess proton motive force [2–8]. Thus during ATP synthesis ADP deficiency seems to stop rotation (cf. our rotating wheel cartoon, Fig. 2).

The effect of this physiological 'state 4 stop' in γ rotation has been seen in mitochondria for a long time; low ADP concentration, i.e. high energy charge, inhibits H^+ efflux and even electron transport, i.e. oxygen consumption. Also in illuminated chloroplasts ADP deficiency prevents H^+ efflux [34], i.e. no uncoupling occurs although proton motive force is highest. It is unknown how under phosphorylating conditions, both in mitochondria and in chloroplasts, the ADP concentration is not brought to below about 0.2 mM [30]. In energized isolated chloroplast thylakoid systems a 'slip' in H^+ efflux in the absence of ADP is observed [35]; how this uncoupling is blocked by small concentrations of ADP (0.2–13 μM depending on the P_i concentration) [35] is also unknown.

A 'state 4 stop' in γ rotation would lead to a stop in F_o rotation and thus prevents H^+ efflux. In agreement, if isolated chloroplasts are illuminated under phosphorylating conditions, the 9-aminoacridine fluorescence quenching, an indicator of ΔpH, is highest without ADP and decreases after addition of ADP (data not shown). We propose that under state 4 condition in chloroplasts and in resting mitochondria with orthodox structure [36] the γ rotation and in turn the catalytic activity is dependent on the ADP concentration, because under physiological conditions γ cannot start rotation and/or β_E cannot close without binding of ADP; either γ or the catalytic interface is a sensor for ADP, which interrupts and re-starts the coupled movements after each 120° step. Thus γ would be a stepper to prevent uncoupling at high energy charge.

During synthesis of each ATP all polar interactions are broken and regained at each 120° step of γ rotation. If at low ADP concentrations the interaction energy of the 12 described catches (Table 1) is larger than the torque accumulated in γ due to F_o rotation, no γ rotation would occur and thus uncoupling and idle movements of β would be prevented; if at higher concentrations of ADP the number/energy of the polar interactions is decreased, enzymatic activity would be allowed to resume when ADP concentration increases under state 3 condition in respiring mitochondria showing condensed structure [36]. ADP may compete and loosen some of the specific polar interactions between subunits γ and β_E. Alternatively it may be possible in future to detect and to calculate (at least to simulate) on a complete F_1 structure whether specific repulsive forces at the catalytic interface prevent the closure of β_E under physiological conditions and physiological torque on γ, if ADP is limiting.

The regulation of catalytic activity and H^+ efflux by chloroplast subunit γ due to interaction with subunit ε, reduction of the disulfide bridge or other means, interpreted to reflect a displacement of γ, is discussed elsewhere [28,37–39], as is the function of subunit $F_1\varepsilon$ in *Escherichia coli* [40]. Recently an

additional regulation of the chloroplast ATPase by a 14-3-3 protein has been described [41]. The relative stop position of subunit γ in crystallized chloroplast CF_1 is still open since γ was unresolved in pdb file 1fx0 and in file 1kmh of the tentoxin-inhibited enzyme [42]. In addition the structure of spinach CF_1 with closed and apparently empty binding sites is symmetric, probably caused by crystallization in the absence of Mg [42].

3.3. MEP and the significance of direction of rotation

ATP binding induces and forces a clockwise γ rotation and the conformational change β_E to β_{TP}; ADP binding would permit the forced counter-clockwise γ rotation and the conformational change β_E to β_{DP}. To explain this difference in specificity Oster and Wang [11,12] introduced the MEP. We include it in the rotating wheel cartoon a little bit clockwise next to the β_E determining contacts (Fig. 2). Molecular dynamic simulation of energy transfer steps has suggested [13] that γM25 transduces energy to the mobile domain of β_E during ATP synthesis at about 20°. Indeed, in files 1bmf and 1e1q, βI390 is situated at γM25 counter-clockwise towards α_{TP}, and βE395 at a distance of 8.6 Å from γR36; in pdb file 1e79, βI390 is situated on top of γM25 and βE395 seen as an H-bonded ion pair with γR36 (Table 1); in structure 1h8e former β_E shows ADP docked to it [18], γM25 has passed the jaw and βE395 is seen as an H-bonded ion pair with γR33. We propose that ATP binding starts with the β_E jaw counter-clockwise of γM25 and that ADP binding starts with the β_E jaw clockwise of γM25; the empty conformations E_T and E_D (Fig. 1d) would be slightly different. The necessary counter-clockwise rotation of γ, to start ATP synthesis, can be driven by F_oc rotation.

The energy transfer by γN234 in the simulation [13] does not correspond to any known conformational change; but another residue, γN238, discovered to transfer energy to β during ATP synthesis at about 90°, seems to fit. Residue γN238 possibly forms an H bridge to βA389/I390 both in 1e1q and 1e79 and in 1h8e (Table 1). Indeed, the structure suggests that this contact, as a second eccentric point, pushes β_{TP} towards α_{DP} to further loosen ATP binding at the interface β_{TP}/α_{TP} after ATP synthesis, but from 0° to about 30° and not at about 90° as found in the simulation [13]. The calculated energy transfer from γN238 to β at about 90° during ATP synthesis might, however, reflect that γN238 pulls the β mobile domain out of the 'catalytic catch' seen in β_{DP} in the presumed transition state in 1h8e (cf. Fig. 2); six polar interactions are reduced to one by this counter-clockwise movement of γ (Table 1), when β_{DP} changes to the TP conformation. In reverse, after ATP hydrolysis, the clockwise movement of γ may be brought about by pulling γ into these six polar interactions with β in the DP conformation (an alternative mechanism of the second power stroke as compared to the proposal in [11,12]).

3.4. Remarks and predictions on mutants

Functionally important residues of γ are not obvious from sequence alignment and analysis of conserved residues. Site-specific mutations can contribute to their identification. In *E. coli* the γM23K mutant is believed to form an H-bonded ion pair with βE381 (EC numbering; corresponds to bovine βE395), because coexpression of βE381Q, A or D restored coupling [43]. Since γM23 is very close to γR75 in the bovine structure, this mutation interferes with the ion pair γR75–βE395 in the 'catalytic catch' [17]. Uncoupling seems to be caused by changes of catches. When acidic residues of DELSDED in the thermophilic *Bacillus* PS3 were mutated to Ala, only the βE381A (bovine E395) mutant was found to be inhibited in ATPase activity by 80% [44], none however in torque generation. Therefore the torque generation seems to be independent of the turnover of the enzyme. According to our hypothesis of the triple-track geared energy transformation from γ to β, mutational effects in DELSEED residues may be overcome by D386 attraction or specific interactions of the hydrophobic residues. No mutation corresponding to βD386 is known to us.

We predict that introduction of additional catches/collisions by double mutation in appropriate positions in the described rails or in the groove on γ and in the jaw of β will slow down catalytic activity in the mode of energy transfer inhibitors, i.e. without uncoupling. The introduction of cysteines, e.g. replacing γN238 and βI390, would prevent the specific interaction in β_{TP}; crosslinking will inhibit γ rotation. We further predict that replacement of the bulky residues γM25/V26, the presumed MEP, by Ala or Gly or replacing βI390 and/or L391 by a small glycine will disturb the ATPase-driven γ rotation. If F_1 subunit γ is accepted to work as a screw conveyer both during ATP hydrolysis and during ATP synthesis, many more new structure-based site-specific mutational studies are suggested. This will contribute to our understanding of the mechanism of energy transduction and coordination of the active sites by subunit F_1 γ, recently identified by Senior et al. [8] as another experimental area ripe for development.

4. Addendum

After submission of this article a second molecular dynamic simulation of movements in bovine F_1-ATPase was published [46], using biased and targeted [47] molecular dynamics. Among the topics of our article, Ma et al. [46] focus on the functional interaction of rotating γ with the barrel of the large subunits. They recognized the significance of the positively charged collar on γ, called an ionic track, and confirm our deduced idea of specific interactions with residues of subunit β, i.e. how γ rotation is geared to β up/down movements during ATP synthesis. The closing motion $\beta_{E\rightarrow DP}$ is simulated to involve primarily electrostatic interactions, while the outward and downward opening $\beta_{TP\rightarrow E}$ is concluded to be mainly due to steric repulsion. Additional postulated interactions remain to be analyzed. Subunit γ can pass the α subunits, which also move, without major collisions, because the helical domain is less extended in the superposition of bovine α with β [46]. In a sequence alignment of αY397–R420 with βY381–R406 we have noticed that the jaw of α is less bulky and shorter by two residues; in the loop Ile is replaced by Ala three times, and a residue functionally corresponding to βD386 is missing.

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