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Hypothesis

The 'non-exchangeable' nucleotides of F_1–F_0ATP synthase

Cofactors in hydrolysis?

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The F_1–F_0 ATP synthase bears 6 nucleotide binding sites, only 3 of which turn over during catalysis. The remaining 3 are occupied by slowly exchanging ATP in vivo, although at least 1 molecule is generally lost on isolation of the enzyme in the absence of nucleotide. It is proposed that the function of the slowly exchanging (NC) nucleotides is to participate in catalysis, the terminal phosphate of the bound ATP acting as an acid catalyst in the cleavage/synthesis of the phosphate anhydride bond in the catalytic sites. Such a role has been demonstrated for the bound pyridoxal phosphate moiety in glycogen phosphorylase. Evidence is presented that (i) the NC nucleotide spans the interface between an α subunit and its partner β, interacting near the catalytic binding site on β; (ii) the phosphate moieties of the catalyzed and NC nucleotide are close in space; and (iii) occupation of the NC nucleotide sites promotes ATP hydrolysis by F_1 or its subfragments. All of these findings are required by the proposed mechanism. Relationships between phosphorylase and F_1 structures are discussed.

ATP synthase; Non-catalytic site; Acid catalyst; Phosphate; Subunit interface

SUBUNIT STRUCTURE OF THE ATP SYNTHASE

The F_1–F_0 complex, or ATP synthase, is the enzyme responsible for ATP synthesis linked to electron transfer in mitochondria, chloroplasts and bacterial membranes. It is separable, by physical methods, into a membrane sector, F_0 (the proton channel), and a soluble protein, F_1 (an ATPase), which bears the sites at which ATP, ADP and P_i interact with the complex [1,2].

F_1 itself has a complex subunit structure, comprising 3 copies of each of 2 large (ca. 50 kDa) subunits arranged in a staggered triangular array, with the larger subunit (α) closest to the membrane (Fig. 1) [3,4]. Single copies of additional, smaller subunits ($\gamma\delta\varepsilon$) lie within the central cavity of this array, and bridge its interaction with the membrane sector, F_0.

Kinetic studies [5–8] are consistent with the presence of 3 catalytic sites on F_1. These interact with each other such that (i) binding to site (n + 1) is weaker than to site n (negative cooperativity in binding), but (ii) catalysis at site n is promoted by binding to site (n + 1) (positive cooperativity in catalysis). This results in an 'alternating site' model for normal catalysis in which the 3 sites operate in turn. This model requires non-equivalence of the 3 catalytic sites at any instant, probably achieved by asymmetrical positioning of the γ subunit (Fig. 1). Over a complete cycle of turnover, all 3 catalytic sites pass through 3 identical conformations but 120° out of phase; this may be accompanied by functional rotation of the γ subunit between catalytic sites.

Turnover by this mechanism, involving all catalytic sites, is termed 'multisite catalysis', and occurs at saturating levels of ATP (or ADP in the synthetic direction). At very low substrate concentrations, it is possible to fill only the first catalytic nucleotide binding site so that ATP binds here, is hydrolysed (slowly) and ADP and P_i leave (very slowly) without any change in subunit interaction. This is termed 'unisite catalysis' [2,8,9], and presumably involves only interactions occurring within a single catalytic site. As such, its study allows us to probe primary catalytic processes within F_1.

THE SEARCH FOR CATALYTIC RESIDUES

Each F_1 molecule, therefore, must bear 3 binding sites for ATP/ADP [10,11] which bind and release nucleo-

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Abbreviations: F_1, soluble portion of the H^+-ATP synthase; EF_1, F_1 from *E. coli*; CF_1, F_1 from chloroplasts; TF_1, F_1 from the thermophilic bacterium, PS3; RF_1, F_1 from *Rhodospirillum rubrum*; NC sites, sites of binding of the non-exchangeable ('non-catalyzed') nucleotides; FSBA/FSBI/FSBεA, 5′-*p*-fluorosulphonylbenzoyl adenosine/inosine/ethenoadenosine; DCCD, dicyclohexylcarbodiimide; Nbf-Cl, 7-chloro-4-nitrobenzofurazan; Ap_4A/Ap_5A, diadenosine tetraphosphate/pentaphosphate; the numbering system used in the text to denote residues in F_1 is based on the sequence numbering of the α and β subunits of EF_1 [25].

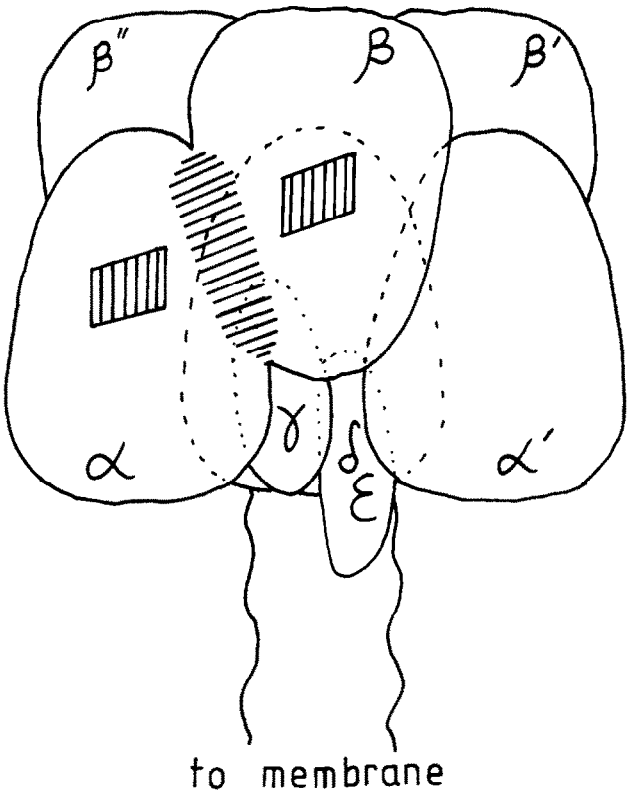

Fig. 1. Model for the F_1 portion of ATP synthase (after Boekema et al. [4]). The 3 α and β subunits are indicated as being slightly different in configuration (α, α′, α″ etc.) due to the asymmetrical position of the γ subunit. On the front face, the functional αβ interface is shaded (unboxed) and 2 putative nucleotide binding sites are shown (boxed and shaded), 1 either side of the interface (see text). Each αβ pair (αβ, α′β′, α″β″) would show such an arrangement, but only the front pair can be seen in this orientation. Note that the αβ′ interface differs from the (functional) αβ interface.

tides during each catalytic turnover. From affinity labelling with analogues such as 2-azido ATP [12], FSBI [13], and chemical modification with reagents such as DCCD [14] and Nbf-Cl [15], these sites are believed to reside on the β subunits of F_1. This is consistent with the presence of 2 nucleotide binding motifs, indicating an ATP binding domain, in the central region of the β sequence [16]. These motifs, the P loop GxxGxGKT (residues 149–156, x = any residue) and the 'Walker motif II' ϕϕϕϕD (residues 238–242, ϕ = hydrophobic residue), are believed to bind to the phosphate moieties of ATP and ADP [17]. Tyr-331 may interact with the adenine moiety [18].

Searches for essential catalytic residues in this domain have, however, been unsuccessful. A plausible mechanism for ATP hydrolysis by this enzyme (which does not involve E–P covalent bond formation) would be the deprotonation of water by an active site base – promoting nucleophilic attack by OH^- – and protonation of the leaving phosphate by a strategically placed acid (Fig. 2a) [19].

The catalytic base might be the anion of a conserved acidic side chain in the ATP binding domain. Mutations at the site of a number of plausible residues in the β subunit from EF_1 (from *E. coli*) have been studied, and are indeed deleterious to catalysis [20–23]; however, even the most incapacitating, E181->Q, lowers catalysis by only 3 orders of magnitude [22]. The nature of the catalytic base thus remains unknown. A similar situation exists for the catalytic acid group. From sequence comparisons, no residues other than Asp or Glu are even candidates for the catalytic acid group, while the participation of Asp or Glu as a proton donor seems unlikely, as the pK_a of the catalytic acid must lie above 8.0 [8,24].

Our inability to identify catalytic residues in F_1 may, of course, have several causes. First, directed mutagenesis studies are difficult to plan and assess because they are as yet conducted in the absence of a detailed molecular structure. Secondly, strong cooperativity during normal (multisite) F_1 turnover leads to the existence of a large class of mutations/modifications that do not affect catalysis directly (unisite catalysis unaltered), but that nonetheless may abolish > 99% activity by disrupting subunit interactions [1,2,25]. Thirdly, F_1 may possess no such primal catalytic groups; instead, catalysis might be a function of several, probably hydrogen bonding, groups, each contributing a small degree of transition state stabilization. Such an arrangement is observed in tyrosine tRNA synthetase, and has been proposed for F_1 [25].

It is the object of the present article to propose a fourth possibility – that the catalytic acid group in F_1 is not, in fact, an amino acid side chain, but a phosphate group of one of the non-exchangeable nucleotides invariably bound to active F_1 preparations. Such a novel role has been recently demonstrated for the phosphate group of bound pyridoxal phosphate in glycogen phosphorylase [26]. This model, besides proposing a mechanism for catalysis on F_1, also provides a rationale for the existence, tightly bound to F_1 in its native state, of ATP/ADP that does not turnover during catalysis – the so-called 'non-catalytic' or 'non-exchangeable' nucleotides of F_1.

'NON-EXCHANGEABLE' NUCLEOTIDE BINDING TO F_1

As noted above, F_1 contains 3 nucleotide binding sites which turnover during catalysis. F_1 from all species tested contains 3 further nucleotide binding sites [11,27]. Nucleotides bound at these sites do not turn over – neither their adenine moiety nor their phosphate group(s) become incorporated into medium nucleotide over prolonged periods. They are known variously as 'tight' [27], 'non-exchangeable' [10] or 'non-catalytic' [11] nucleotide binding sites. None of these terms is entirely satisfactory: (i) ATP can bind very tightly (K_d

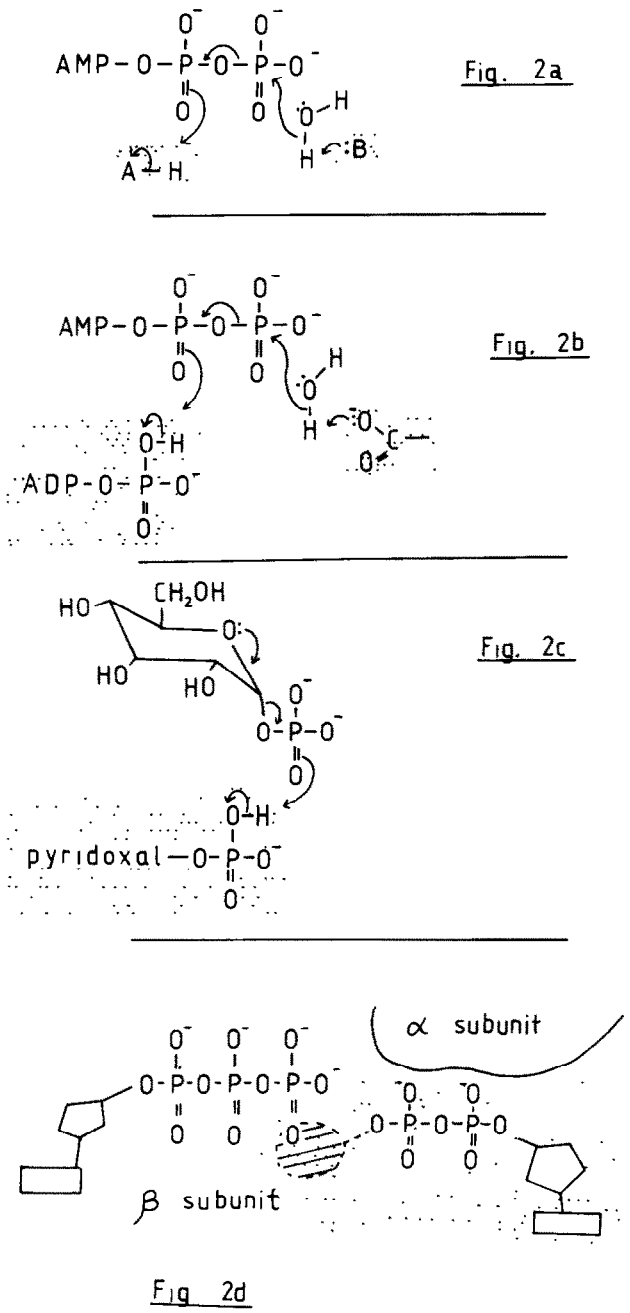

Fig. 2. Acid catalysis by enzyme-bound phosphate. (a) Acid/base catalysis of ATP hydrolysis (after Knowles [19]). (b) Postulated mechanism for F_1, showing tightly bound ATP as acid catalyst, and carboxylate residue as base (this paper). (c) Initial step in glucose-1-phosphate reaction with glycogen, catalyzed by glycogen phosphorylase (after Johnson et al. [56]). (d) Orientation of ATP in C site and ADP in NC site as proposed by Vogel and Cross [2,53]. A possible site for an additional phosphate on the NC nucleotide (forming ATP) is shaded. Dotted areas indicate enzyme-bound (catalytic) moieties: reactants are shown open.

$< 10^{-10}$ M) to the catalytic sites of F_1 [2,11]; (ii) exchange of 'non-exchangeable' nucleotides *can* occur [27,28], but over periods of minutes as compared to the millisecond time scale of catalysis [29,30]; and (iii) I intend in the present article to propose a role for nucleotides bound to these sites in the catalytic mechanism of F_1. For consistency, however, I shall follow the nomenclature of Kironde and Cross [11] and denote these as NC (for *non-catalyzed*) nucleotide binding sites. Sites at which nucleotides turn over during the catalytic cycle are denoted C binding sites.

Washed membrane-bound F_1, from all species tested, has 2 of its 3 NC sites occupied by ATP [27]. This value is so consistent that the F_1 content of coupling membranes can conveniently be estimated from their 'tight' ATP content (retained after washing with pyrophosphate) [31,32]. Soluble F_1 may also contain 2 ATP bound at NC sites per mole F_1 [11,27], but some ATP may hydrolyse to ADP or be lost on storage. TF_1 (from the thermophile, PS3) can be easily obtained with all NC sites empty [33], but this is exceptional – depletion of mitochondrial F_1 from all NC ATP involves prolonged gel filtration in 50% glycerol [34].

Membrane-bound or soluble F_1 also commonly contains 1 mol slowly exchanging ADP per mole enzyme [27]. However, this ADP is bound at a C site; its release from this site is abnormally slow due to premature loss of its normal companion, P_i [35]. Unlike nucleotide at the NC sites, ADP at the C site blocks normal turnover, and must be released from an F_1 molecule before it is catalytically active [28,35].

In summary, a particularly stable state of mesophilic F_1 has 2 (of 3) NC sites filled with ATP, and ADP fortuitously trapped at one (of 3) C sites. This can be denoted $F_1(2,1)$, where the first digit indicates the number of NC sites occupied, and the second, the number of C sites occupied (after [11]). Nucleotide-replete F_1, such as presumably occurs under saturating turnover conditions, would be designated $F_1(3,3)$. The isolation of F_1 with some, but less than all, nucleotide binding sites filled is due to unequal affinities at the various sites (negative cooperativity of binding).

One operational distinction between the 2 classes of binding site lies in their nucleotide specificities [11,36,37]. C sites (at least in their lowest affinity conformation) show affinities for GTP and ITP within an order of magnitude of ATP. NC sites show a much stronger preference for adenine nucleotides. However, recently it has been shown that GTP can occupy these sites, and form a stable, slowly exchanging complex [38]. Both these sites seem to tolerate modification of the ribose ring of the nucleotide [1,2,25].

ROLE OF THE NC NUCLEOTIDES – AN HYPOTHESIS

Since their discovery in 1973 [39], the role of the NC nucleotides has been enigmatic. Originally, tightly bound NC ATP was suggested to represent a low energy intermediate in ATP synthesis [39], as later incorporated into the 'binding change' model for ATP synthesis

[40]. However, while this model persisted, participation of the NC nucleotides as intermediates in turnover was ruled out by kinetic studies [29,30]. Models involving transphosphorylation between C- and NC-bound nucleotides were similarly eliminated (see e.g. [41]). Further suggestions have included a regulatory role (unlikely due to their high affinity for nucleotide), a structural role, and, recently, a presence as an evolutionary relic [42]. While difficult to disprove, none of these suggestions has significant experimental support.

I propose here that NC nucleotide acts as an acid/base catalyst during ATP hydrolysis/synthesis. Fig. 2b shows a possible mechanism of ATP hydrolysis, in which phosphate from an NC nucleotide acts as a proton donor to the phosphate of ATP at a C site. The latter thus has its susceptibility to nucleophilic attack by H_2O/OH^- increased, and hydrolysis is catalyzed. This mechanism involves NC nucleotide as a *cofactor* in catalysis.

The use of bound phosphate, rather than an amino acid side chain, as an acid catalyst is unusual, but not unprecedented. A similar role has been established for the phosphate of bound pyridoxal phosphate in glycogen phosphorylase, and this mechanism is reproduced in Fig. 2c. There may be some kinetic advantage in transferring H^+ between 2 phosphate groups rather than between phosphate and a different base; alternatively such a mechanism may reflect an evolutionary relic of prebiotic catalysis by inorganic materials. Whatever the reason, this mechanism provides a rationale for the presence of NC nucleotide on F_1, and for the inability to identify catalytic amino acid side chains. Two predictions follow from this model; (i) that the C and NC binding sites must be close together in space, and (ii) that the presence of nucleotide at NC sites is a requirement for catalysis by F_1. These predictions are discussed below.

LOCATION OF THE NC BINDING SITES

A considerable body of work implicates the α subunits of F_1 in binding NC nucleotides. It was originally pointed out by Harris [43] that the α and β subunits of F_1 were homologous polypeptides, and Walker and coworkers [44] went on to identify nucleotide binding motifs, the P loop and 'Walker motif II' (see above) within the α subunit. Furthermore, isolated α subunit from EF_1 or TF_1 binds nucleotide with high affinity [45,46], which is reduced on mutating this subunit [47]. Finally, the affinity labels, PLP-AMP and FSBεA label holo F_1 within the predicted nucleotide binding domain of the α subunit (residues 160–330) [48,49] when bound at an NC nucleotide binding site. It is therefore tempting to place each of the 3 NC nucleotide binding sites on an α subunit, by analogy with positioning each C binding site on a β subunit (Fig. 1).

However, the true situation is probably not so simple.

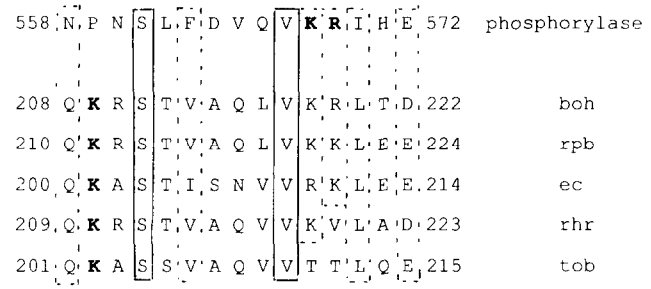

Fig. 3. Peptide sequences from phosphorylase and F_1. The upper sequence is a pentadecapeptide from the catalytic region of phosphorylase [66], and includes the phosphate binding residues K568 and R569, shown bold (see text). The lower sequences are taken from the α subunits of various species of F_1 [44], with the site of affinity labelling with PLP-AMP (K201), shown bold. Identities are boxed with full lines, conservative replacements in dotted lines. boh, bovine heart; rpb, *Rhodopseudomonas blastica*; ec, *E. coli*; rhr, *Rhodospirillum rubrum*; tob, tobacco chloroplast.

It seems likely that β subunits also participate in NC nucleotide binding. Some affinity labels, notably FSBA and 2-azido ATP, preferentially label the β subunits when bound to NC sites [12,13]. Other affinity labels (8-azido ATP or 8-azido FSBA) at NC sites can label both α and β subunits [50,51]. All these findings can be rationalized only if the NC binding sites occupy $\alpha\beta$ interfaces, instead of being localised on individual α subunits.

Other evidence for NC binding at an $\alpha\beta$ interface comes from recent work on the reconstitution of a functional $\alpha\beta$ heterodimer from isolated α and β subunits of RF_1 (from *Rhodospirillum rubrum*) [52]. It was shown that a non-exchangeable nucleotide binding site is formed when α and β subunits associate to form a dimer, whereas no such site is present on individual subunits.

It is thus feasible that both C and NC binding sites are close together on F_1, with the NC nucleotide spanning an $\alpha\beta$ interface towards the C binding site on a β subunit. Experimental evidence for proximity of the 2 sites comes from the position of NC-site labelling with FSBA and 2-azido ATP. These reagents label Tyr-354 [12,13], only a few residues upstream from residue Tyr-331, which has been implicated in C-site binding [2].

The model proposed in Fig. 2b specifically predicts proximity of the *phosphate* groups of nucleotides bound at the C and NC sites. Unfortunately, reconstitution studies on the $\alpha\beta$ heterodimer cannot provide information on the orientation of the 2 binding sites, while the above labelling studies reveal only residues close to the adenine ring. However, Vogel and Cross [53], working with the diadenosine phosphates Ap_4A, Ap_5A etc., have shown that these compounds, with suitably spaced adenosines, are as powerful inhibitors of F_1 as they are of adenylate kinase. Furthermore, inhibition occurs only

if F_1 has at least 1 NC and 1 C site unoccupied, indicating that these inhibitors must bridge C and NC sites. This would imply that the phosphate groups of C and NC nucleotides (which are linked in these analogues) are, indeed, in close proximity in functioning F_1 as the proposed model requires (Fig. 2d; cf. [2]). This conclusion is supported by the demonstration of spin–spin interaction between spin-labelled ATP bound at the 2 sites [54].

REQUIREMENT OF NC NUCLEOTIDE FOR F_1 ACTIVITY

It has proved surprisingly difficult to obtain a definitive answer as to whether or not NC nucleotide is required for catalysis by F_1. There are 2 major problems. First, no procedure is available for stripping tightly bound ATP from any preparation of membrane-bound F_1. Thus, effects of NC ATP on ATP synthesis can be studied, after considerable manipulation, only in F_1-depleted, then reconstituted, membranes. Secondly, assay of activity of nucleotide-depleted, soluble F_1 (either in the direction of ATP hydrolysis or synthesis) requires nucleotides to be present, with the likelyhood that any empty nucleotide sites will refill during the assay (cf. [55]). In addition, the requirement for high glycerol concentrations in most procedures for depleting F_1 of NC nucleotides [34,37] is problematic, since glycerol interferes with assay of nucleotide using A_{260}/A_{280} ratios [56,57] or by luciferase (Harris, D.A., unpublished). In other words, some preparations of 'nucleotide-depleted' F_1 reported in the literature may still contain bound ATP.

Early experiments indicated a correlation between tightly bound nucleotide content and enzyme activity of MF_1 [58,59], when nucleotide was removed by treatments such as cold denaturation. It was concluded that NC-bound ATP was required for catalysis. Interpretation of such experiments is, however, somewhat equivocal because the F_1 used was initially (probably) in the $F_1(2,1)$ state (see above), with inhibitory ADP also bound at a C site [27]. More convincing is the recent demonstration that NC nucleotide binding to an $\alpha\beta$ interface leads to a 10^2–10^3-fold stimulation compared to hydrolysis by the β subunit alone [52]. These experiments employed an $\alpha\beta$ heterodimer derived from RF_1, in which (i) the nucleotide site occupation could be readily established, and (ii) cooperativity between the 3 $\alpha\beta$ dimers in the (hexameric) holo F_1 structure was abolished. Under these conditions, the interactions between 1 NC site and its companion C site could be directly assessed.

In contrast, a series of experiments (largely by Senior and co-workers) have been taken to suggest that NC nucleotides are not required for catalysis [37,60–62]. In these experiments, EF_1 or MF_1 were depleted of nucleotides, typically by gel filtration in 50% glycerol, but were shown nonetheless to be capable of NuTP hydrolysis or NuDP phosphorylation. Inosine or guanosine nucleotides were used in the assays, to preclude refilling the NC binding sites, which show a high specificity for adenine nucleotides (above). However, while ITP/GTP do not show the very tight binding observed with ATP, it appears that they can occupy the NC sites at the concentrations used in these assays [38]. In addition, some NC adenine nucleotide may still be present in glycerol-treated preparations (see above, [63]). Thus, these experiments cannot conclusively eliminate a role for NC nucleotide in catalysis.

In an alternative approach, an α subunit mutant of EF_1, which had decreased ATP binding to isolated α subunit, was shown to be still able to hydrolyse ATP at 30% of normal rates [47]. Again, however, the nucleotide content of holo F_1 in this mutant was not determined, and the presence of NC nucleotide, the binding site of which also involves the β subunit, cannot be unequivocally ruled out.

A recent series of experiments, designed specifically to test the requirement for NC nucleotide, suggested that such nucleotides are indeed necessary in catalysis by CF_1. Boyer and co-workers [38,55,64] correlated the time–course of ATP binding to NC sites in CF_1 (depleted of nucleotide by heating in the absence of ATP) with appearance of ATPase activity. They concluded that ATP at all 3 NC sites was essential for normal catalytic turnover, and that occupation of at least 2 NC sites was required for any activity. Thus, in this species at least, occupation of NC sites by ATP seems essential for catalysis, as predicted by the proposed model. Zhuo et al. [51] refer to similar findings with MF_1.

STRUCTURAL SIMILARITIES BETWEEN GLYCOGEN PHOSPHORYLASE AND F_1

The proposed mechanism for F_1-ATPase uses phosphate bound to cofactor (nucleotide) as a catalytic group in general acid catalysis. The model was developed by analogy with the known mechanism of glycogen phosphorylase, where the cofactor is pyridoxal phosphate. It might be expected, therefore, that the environment of the substrate and cofactor phosphate groups in both enzymes would be similar.

A detailed comparison between the active sites of phosphorylase and F_1 is not yet possible since no high resolution structure of F_1 is known, and the 2 primary structures do not show extended homologies. However, there are 2 intriguing observations that may point in the direction of structural similarity. First, at the level of supersecondary structure, phosphorylase is known to have a nucleotide binding (Rossmann) fold [65], and a P loop structure [26] at its active site, even though it does not bind nucleotide. This structural similarity may point to some analogy in mechanism.

Secondly, at the primary structure level, a sequence

similar to the phosphorylase active site can be detected in F_1. In phosphorylase, the cofactor and substrate phosphate are bound by adjacent residues, Lys-568 and Arg-569 [26]. As shown in Fig. 3, the sequence around these residues, which is conserved absolutely in all known phosphorylase sequences, shows homology with a region in the α subunit of F_1 from both mitochondria and bacteria. This sequence flanks Lys-201, which is the residue modified by PLP-AMP [48], and lies in the centre of the NC nucleotide binding region (above). Crystallographic studies on heavy metal derivatives of the nearby residue, αCys-193 [67] indicate that, as predicted in this model, this region of homology with phosphorylase lies at a subunit interface in F_1.

In the absence of a detailed structure, such sequence comparisons must be treated with caution. Furthermore, while general features of this sequence are conserved between F_1 species, conservation of the basic residues, corresponding to Lys-568 and Arg-569 is not absolute: one is missing in RF_1, and both in CF_1 (Fig. 3). Nonetheless, in view of the evolutionary distance between the F_1 and phosphorylase enzymes, the similarity in sequence observed at these functional sites is noteworthy.

Because of large differences in overall structure, a precise 1:1 correspondence of active site residues between F_1 and glycogen phosphorylase is not, and probably will never be, possible. However, the above evidence points strongly in the direction of a commonality in the participation of cofactor phosphate in (acid/base) catalysis. This model presents a new framework for consideration of the mechanism of F_1, which is intended to aid in the design and interpretation of further structural and functional studies on the enzyme.

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