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Rotation of subunits during catalysis by *Escherichia coli* F_1-ATPase

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ABSTRACT **During oxidative and photo-phosphorylation, F_0F_1-ATP synthases couple the movement of protons down an electrochemical gradient to the synthesis of ATP. One proposed mechanistic feature that has remained speculative is that this coupling process requires the rotation of subunits within F_0F_1. Guided by a recent, high-resolution structure for bovine F_1 [Abrahams, J. P., Leslie, A. G., Lutter, R. & Walker, J. E. (1994) *Nature (London)* 370, 621–628], we have developed a critical test for rotation of the central γ subunit relative to the three catalytic β subunits in soluble F_1 from *Escherichia coli*. In the bovine F_1 structure, a specific point of contact between the γ subunit and one of the three catalytic β subunits includes positioning of the homolog of *E. coli* γ-subunit C^{87} (γC87) close to the β-subunit ^{380}DELSEED^{386} sequence. A βD380C mutation allowed us to induce formation of a specific disulfide bond between β and γC87 in soluble *E. coli* F_1. Formation of the crosslink inactivated βD380C-F_1, and reduction restored full activity. Using a dissociation/reassembly approach with crosslinked βD380C-F_1, we incorporated radiolabeled β subunits into the two noncrosslinked β-subunit positions of F_1. After reduction of the initial nonradioactive β–γ crosslink, only exposure to conditions for catalytic turnover results in similar reactivities of unlabeled and radiolabeled β subunits with γC87 upon reoxidation. The results demonstrate that γ subunit rotates relative to the β subunits during catalysis.**

F_0F_1-ATP synthases are found embedded in the membranes of mitochondria, chloroplasts, and bacteria and show general structural/functional conservation across species. The F_0 sector is composed of membrane-spanning subunits and is responsible for transport of protons across the bilayer. The F_1 sector is an extrinsic complex that contains the catalytic sites for ATP synthesis and hydrolysis. F_1 can be removed from the membrane in a water-soluble form that catalyzes ATP hydrolysis. F_1 has the subunit composition $\alpha_3\beta_3\gamma\delta\varepsilon$ in which a hexamer of alternating α and β subunits surrounds the central γ subunit. The catalytic nucleotide sites are located on the β subunits at α/β-subunit interfaces (reviewed in refs. 1–3).

A model for energy coupling by F_0F_1, called the binding change mechanism (3, 4), has two features that are widely accepted (Fig. 1*a*). The first is that the major energy-requiring step is not synthesis of ATP at the catalytic site but rather its release from the site (8). Second, the tight binding of substrates and release of product occur simultaneously at separate but interacting sites (9). A third premise has been more speculative: that the required binding changes are coupled to proton transport by rotation of a complex of subunits extending through F_0F_1 (10). Rotation of the γ subunit in F_1 is thought to deform the catalytic sites to give the binding changes (Fig. 1*a*), whereas rotation within F_0 is believed to be required for completion of the proton pathway (Fig. 1*b*). Evidence both for (5, 10–15) and against (16–19) subunit rotation has been presented, but no critical test has been reported. However, such a test now seems possible in light of a recent, high-resolution structure for bovine F_1 (14). Specific points of contact between γ and the three catalytic β subunits that encircle it include positioning of the bovine homolog of *Escherichia coli* γ-subunit C^{87} (γC87) close to one of the β-subunit ^{380}DELSEED^{386} sequences. In preliminary studies, we identified a Cys mutation within this β-subunit sequence, βD380C, that allowed rapid and specific crosslinking of β to γ subunit on membrane-bound F_0F_1 and concomitant inactivation of ATP hydrolysis and ATP-driven proton pumping (20). In this study, we induced a specific disulfide between βD380C on one β subunit and γC87 in soluble F_1 (Fig. 1*c*, step 1). Then, by subunit dissociation/reconstitution, we incorporated radiolabeled β subunits specifically into the two noncrosslinked β-subunit positions of reconstituted hybrid F_1 (Fig. 1*c*, step 2). After reduction of the initial nonradioactive β–γ crosslink, this allowed us to test the effects of ligand binding and catalysis on the ability of γ subunit to reposition itself relative to specific β subunits (Fig. 1*c*, step 3). Our results show that, after catalytic turnover of reduced hybrid F_1, unlabeled and radiolabeled β subunits show a similar capacity to form a disulfide with γC87, as expected for a rotary mechanism.

MATERIALS AND METHODS

Purification of Soluble *E. coli* F_1. *E. coli* F_0F_1 was overexpressed from wild-type or mutant forms of plasmid p3U (20) and membranes were isolated as described (21, 22). To prepare ^{35}S-labeled βD380C/γC87S-F_1, 2 liters of culture was grown with 10 mCi (1 Ci = 37 GBq) of ^{35}S-labeled Met/Cys (Expre^{35}S^{35}S; DuPont/NEN). Purification of soluble F_1 from membranes (23) was done at 4°C with the following modifications. Precipitation by polyethylene glycol 8000 was omitted. Crude soluble F_1 was applied directly to Productiv DE anion-exchange cartridges (bps separations, San Diego) at a flow rate of 2–4 ml/min and, when necessary, recirculated to achieve maximal loading. The cartridges were washed at 2 ml/min with 3 vol of column buffer alone [50 mM Tris·HCl/40 mM 6-aminohexanoic acid/10% (vol/vol) glycerol/2 mM EDTA/1 mM ATP/1 mM dithiothreitol (DTT), pH 7.2 at 22°C] and then with 3 vol of buffer with 50 mM NaCl. F_1 was eluted at 1 ml/min with buffer containing 0.25 M NaCl. F_1 was concentrated by ammonium sulfate precipitation and then passed through a Sephacryl S300HR column [1 cm (i.d.) × 45 cm for <10 mg of F_1 or 1.5 cm (i.d.) × 65 cm]. Purified F_1 was concentrated to >5 mg/ml by ultrafiltration (Centricon-10; Amicon) and stored at −70°C.

Treatment of F_1 with Oxidizing Agents. F_1 samples were diluted to 1–2 mg/ml with MTKE buffer (20 mM Mops Tris/50 mM KCl/0.1 mM EDTA, pH 8.0), desalted by passage through centrifuge columns of Sephadex G50 (24) equilibrated with MTKE buffer, and diluted to 0.5 mg/ml with MTKE buffer for

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol.

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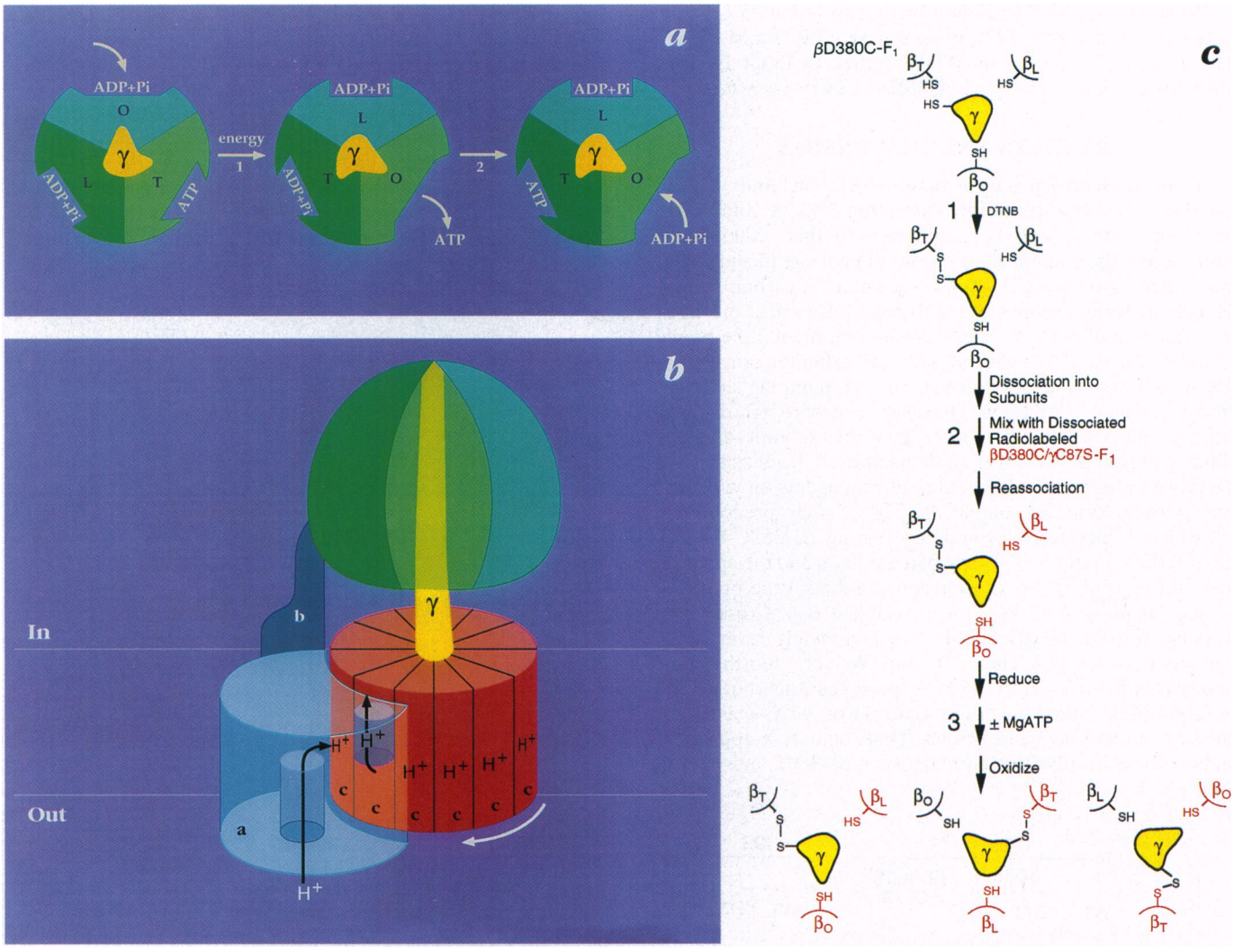

FIG. 1. The binding change mechanism. (*a*) View from the top of F_1. In step 1, rotation of the asymmetric γ subunit forces conformational changes in the three catalytic sites, which result in affinity changes for substrates and product. T, L, and O, tight, loose, and open conformations, referring to the decreasing affinities of catalytic sites for ligand. Each of the three blue/green areas represents a pair of α and β subunits where the catalytic sites are interfacial but mostly on β subunits. In this illustration, the $\alpha\beta$ pairs remain stationary. In step 2, ATP forms spontaneously from tightly bound ADP and P_i. (*b*) View from the side of F_0F_1. The arrangement of F_0 subunits is based on refs. 5–7. Subunit a contains two partial channels, each in contact with a different side of the membrane. In order for a H^+ to traverse the membrane, it enters one channel, moves to the center of the membrane, transfers to one of the c subunits, and is then carried to the other partial channel by rotation of the complex of c subunits. The c subunits are anchored directly or indirectly to γ subunit, whereas subunit a is anchored, probably indirectly through subunit b, to the catalytic subunits. Hence, the rotation of c subunits relative to subunit a in F_0 will drive the rotation of γ subunit relative to the catalytic subunits in F_1. (*c*) An experiment designed to test the rotary feature of the binding change mechanism.

incubation at 22°C with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) or 2-iodosobenzoate. After treatment, aliquots were removed for assays of ATPase and protein and for SDS/PAGE.

Dissociation of F_1 and Reconstitution of F_1 Hybrids. Separate F_1 samples (with or without pretreatment with DTNB) were passed through centrifuge columns equilibrated with MTKE buffer and then precipitated with 2 vol of saturated ammonium sulfate. After centrifugation, F_1 pellets were dissolved in dissociation buffer (50 mM Mes·NaOH/1 M LiCl/5 mM ATP/0.5 mM EDTA, pH 6.1) and dissociated by two freeze/thaw cycles as described (25). ATPase assays were done (with DTT for DTNB-treated samples) to confirm that dissociation was maximal. Dissociated samples were combined as described (see Fig. 3 legend), immediately diluted to 0.5 mg of F_1 per ml with reconstitution buffer (50 mM Mes·NaOH/10% glycerol, pH 6.0) lacking MgATP, and then dialyzed (Slide-A-Lyzer 10K; Pierce) for 10–12 hr at 22°C against reconstitution buffer containing 2.5 mM $MgCl_2$ and 2.5 mM ATP. Each sample was then concentrated to ≥1.5 mg/ml by ultrafiltration (Spin-X UF10; Corning–Costar). Hybrid F_1 samples previously treated with DTNB were then treated with 0.2 mM *N*-ethylmaleimide for 25 min (this had no significant effect on ATPase activity of hybrid F_1 assayed after reduction with DTT). Each reconstituted F_1 sample was then passed through a centrifuge column equilibrated with MTKE buffer and aliquots were used for the experimental treatments described in the legend for Fig. 3.

Electrophoresis. SDS/PAGE (26) used 4–15% gradient gels (Ready Gels; Bio-Rad) and 0.5 mM *N*-ethylmaleimide was added to the gel sample buffer instead of 2-mercaptoethanol. "Mark-12" protein standards were from NOVEX (San Diego) and ^{14}C-labeled protein standards were from GIBCO/BRL. Gels were stained with Coomassie blue R250 and relative amounts of β- and γ-subunit bands and the 86-kDa band were determined by photodensitometry. For detection of radiolabeled bands, dried gels were analyzed with a PhosphorImager (model 425E; Molecular Dynamics).

Other Assays. ATP hydrolysis was measured at 25°C with a coupled-enzyme assay (27), using 5 mM ATP, 2 mM $MgCl_2$, and 1 μg of F_1 per ml in MTKE buffer (without EDTA). Protein was determined by a modified Lowry assay (28).

RESULTS AND DISCUSSION

Disulfide Bond Formation Between βD380C and γC87 in Soluble F_1. Previously, we introduced the βD380C and γC87S mutations into *E. coli* F_0F_1 and showed that either single mutation or the combined mutations did not significantly alter phenotypic growth of cells with succinate as carbon source. Hence, these mutations appear to have little effect on the *in vivo* function of F_0F_1. We also showed that membrane-bound βD380C-F_0F_1 and βD380C/γC87S-F_0F_1 exhibited normal levels of ATPase and ATP-driven proton pumping activities under reduced conditions. However, exposure to oxidizing agents induced formation of a β–γ intersubunit disulfide linkage only in βD380C-F_0F_1 and inactivated the enzyme (20). Here we examine the effects of oxidizing agents on wild-type and mutant forms of soluble F_1 (Fig. 2). One predominant crosslinked species is formed by treating βD380C-F_1 with DTNB (lane 2) or 2-iodosobenzoate (lane 7), and the apparent size of the product (86 kDa) is consistent with the predicted molecular mass of 82 kDa for crosslinked β–γ. Crosslinking and inactivation of βD380C-F_1 are completely reversed by reduction with DTT (lane 3), and Western blotting (not shown) confirmed that the 86-kDa species contains both β and γ subunits. If either the β-Cys (lane 1) or γ-Cys (lane 4) is missing, no 86-kDa band is seen. Thus, oxidation appears to induce a specific disulfide bond between βD380C and γC87 in βD380C-F_1.

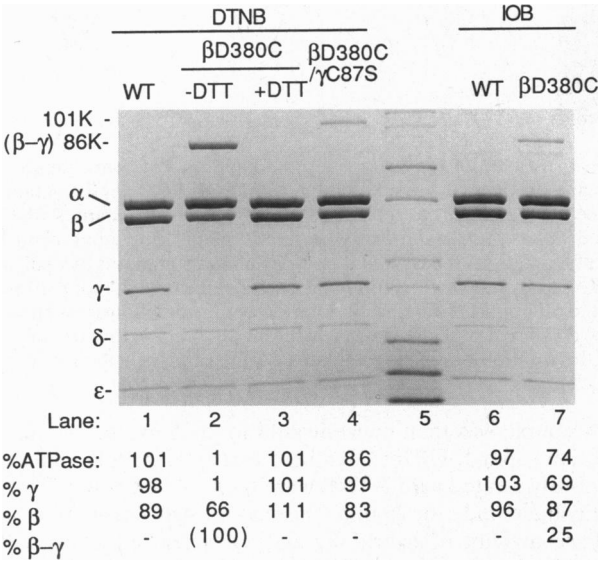

FIG. 2. Reversible inactivation of βD380C-F_1 by formation of a specific β–γ intersubunit disulfide bond. Wild-type (WT) and mutant F_1 samples were treated with 25 μM DTNB or 1 mM 2-iodosobenzoate for 15 min at 22°C and aliquots were taken for SDS/PAGE and ATPase assays. For lane 3, a second 15-min incubation with 5 mM DTT followed reaction with DTNB. Positions of F_1 subunits are noted, along with sizes (kDa) for two higher molecular mass bands that are present only after oxidation of βD380C-F_1 (86 kDa) or βD380C/γC87S-F_1 (101 kDa). Each sample's ATPase activity is listed as a percentage of the activity of an untreated control. ATPase of untreated F_1 (μmol·min^{-1}·mg^{-1}): WT, 34; βD380C, 28; βD380C/γC87S, 23. Amounts are listed for γ- and β-subunit bands in each lane (relative to average of controls) as well as the relative amount of the 86-kDa cross-linked band for βD380C-F_1 treated with DTNB (100%; lane 2) vs. 2-iodosobenzoate (IOB) (lane 7).

Data tabulated at the bottom of Fig. 2 show a good correlation between the percentage of residual ATPase activity, the relative decrease in the bands for β and γ subunits, and the relative amount of the β–γ crosslinked product formed for each sample. For example, in lane 2, there is a 99% loss of both ATPase activity and γ subunit. As expected, this correlates to a loss of 1/3rd of the β subunit. In lane 7, a 26% loss of ATPase activity correlates to the formation of 25% as much of the β–γ product as that formed with near-complete inactivation (lane 2). This indicates that formation of the βD380C–γC87 disul-

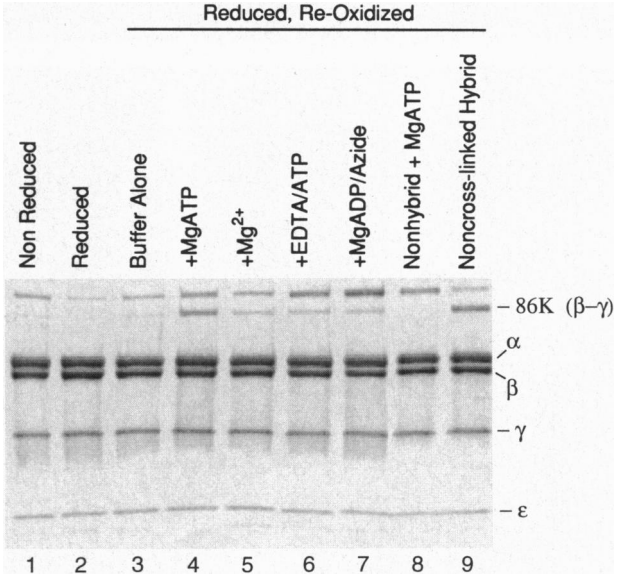

FIG. 3. Use of hybrid F_1 to test for rotation of γ subunit relative to β subunits. The image shows PhosphorImager detection of ^{35}S-containing bands on SDS/PAGE. Positions of the 86-kDa β–γ band and F_1 subunits are indicated on the right; the δ subunit is not clearly visible because it migrates as two bands because of an internal disulfide as noted (29). For samples in lanes 1–7, hybrid F_1 was reconstituted from an equimolar mixture of two dissociated F_1 samples: βD380C-F_1, which was treated with DTNB before dissociation to induce a βD380C–γC87 disulfide bond, plus ^{35}S-βD380C/γC87S-F_1, which was treated with DTNB before dissociation to block βD380C from reaction with other sulfhydryls in the dissociated mixture. Hybrid F_1 was subjected to the following treatments before SDS/PAGE. Lane 1, the original nonradioactive βD380C–γC87 disulfide was not reduced, but the hybrid was treated directly with 25 μM DTNB for 10 min. Lane 2, hybrid F_1 was reduced by exposure to 10 mM DTT for 1 min and then passed through a centrifuge column equilibrated with MTKE buffer; Coomassie staining of the gel confirmed that the original β–γ disulfide was reduced. All remaining samples were treated as in lane 2, except that the buffer on the centrifuge column contained additional components. Lane 3, no additions; lane 4, 0.5 mM $MgCl_2$ + 1 mM ATP; lane 5, 0.5 mM $MgCl_2$; lane 6, 2 mM EDTA + 1 mM ATP; lane 7, 0.5 mM $MgCl_2$ + 0.5 mM NaN_3 + 0.1 mM ADP; lane 8, 0.5 mM $MgCl_2$ + 1 mM ATP; lane 9, no additions. Finally, samples in lanes 3–9 were treated with 25 μM DTNB for 10 min immediately after column centrifugation to induce maximal crosslinking of βD380C to γC87. Sample in lane 8 was a nonhybrid control in which the two DTNB-treated enzymes (βD380C and ^{35}S-βD380C/γC87S) were reconstituted separately before mixing in a 1:1 ratio. Sample in lane 9 was a noncrosslinked control in which a mixture of βD380C-F_1 and ^{35}S-βD380C/γC87S-F_1 was reconstituted without prior treatment with DTNB. Samples were loaded to give the same total ^{35}S per lane (≈36,000 cpm by liquid scintillation counting), which corresponded to ≈6 μg of total protein per lane. The amounts of ^{35}S-labeled α, γ, and ε subunits varied by ≤10% between lanes, and differences in ^{35}S in β-subunit bands were consistent with the amounts of ^{35}S-labeled 86- and 101-kDa bands in each lane (quantitation not shown). By Coomassie staining of the gel (not shown), the total amount of 101-kDa band is significantly less than the amount of 86-kDa band in each case except for lane 7. The ^{35}S in 101-kDa bands is exaggerated in part because it can contain up to two radiolabeled subunits.

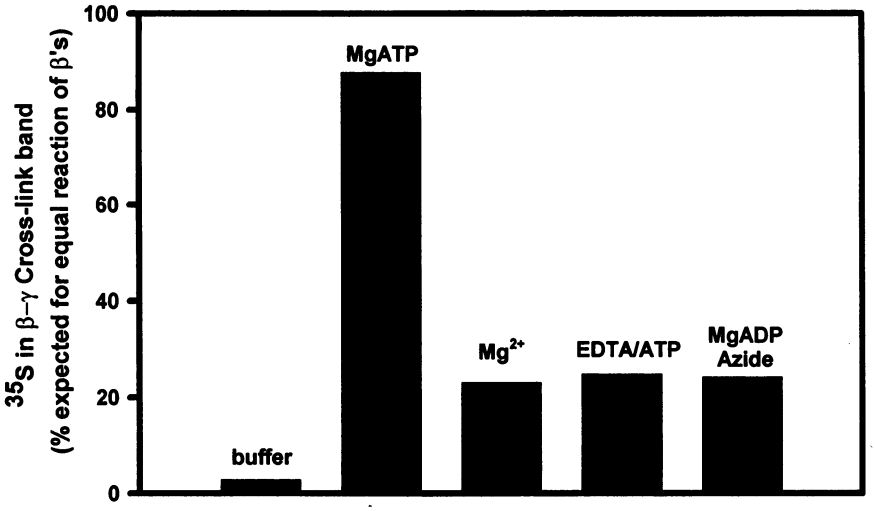

FIG. 4. Quantitation of ^{35}S-labeled β subunit in the 86-kDa β–γ band of hybrid F_1 after reduction and reoxidation. A small amount of ^{35}S in the 86-kDa band in lane 1 of Fig. 3 represents disulfide exchange that apparently occurred during reconstitution, and this value was subtracted from all other values for comparisons of ^{35}S incorporated into the 86-kDa band after reduction and reoxidation. The amount of ^{35}S in the 86-kDa band in lane 9 (Fig. 3) represents random crosslinking of γ subunit to labeled and unlabeled β subunits within F_1. After correcting for the statistical difference in ^{35}S-labeled β-subunit content for reconstituting hybrid F_1 with or without an initial, nonradioactive β–γ crosslink (a factor of 0.8) and subtracting background (lane 1, ^{35}S-labeled 86-kDa band), the resulting value was set equal to 100% for comparison with ^{35}S-labeled 86-kDa band observed after reduction and reoxidation of previously crosslinked hybrid F_1 (see Fig. 3, lanes 3–7). If γC87 were to rotate between the original unlabeled β subunit and only one of the two labeled β subunits, the anticipated level of ^{35}S in the β–γ crosslinked product would be 25% less than that for random exchange between all three β subunits.

fide is directly responsible for inactivation of βD380C-F_1. Recently, inactivation of F_1 by β–γ crosslinking was also shown for other Cys mutants in the DELSEED region of β subunit (12).

A minor crosslinked species (101 kDa) is observed with oxidation of βD380C/γC87S-F_1 but not with wild-type F_1 or βD380C-F_1 (Fig. 2, lane 4 vs. lanes 1 and 2). Western blotting (not shown) shows that this 101-kDa species contains β subunit but does not contain the γ subunit. Hence, it may be a β–β dimer (predicted β–β, 100.6 kDa).

Use of Reconstituted Hybrid F_1 to Examine Interaction of γC87 with Distinct β Subunits in F_1. Preliminary studies (not shown) confirmed that DTNB-treated βD380C-F_1 can be dissociated and successfully reconstituted without breaking the βD380C–γC87 disulfide bond. The reconstituted enzyme regains activity immediately upon reduction. This then provides a means of introducing radiolabeled βD380C in place of the two non-crosslinked β subunits (Fig. 1*c*, step 2) to allow the nonradiolabeled β subunit initially crosslinked to γ subunit to be distinguished from β subunit in the other two positions. For this purpose, DTNB-treated βD380C-F_1 was dissociated, mixed in a 1:1 molar ratio with dissociated ^{35}S-labeled βD380C/γC87S-F_1, and reassociated. In addition to the desired hybrid F_1 (Fig. 1*c*, step 2), about half the enzyme molecules will contain radiolabeled γC87S. However, F_1 containing γC87S is incapable of forming the 86-kDa β–γ crosslink (although this F_1 will give the 101-kDa band, as shown in Fig. 2, lane 4). As a test for rotation, the hybrid F_1 was then reduced, exposed to different ligand conditions, and reoxidized (Fig. 1*c*, step 3). The question asked is whether γC87 crosslinks only to its original, nonradioactive partner or, if γ subunit has rotated relative to the β subunits, ^{35}S will now appear in the 86-kDa band. Fig. 3 shows the distribution of ^{35}S-labeled bands on SDS/PAGE for a typical experiment, and the relative amounts of ^{35}S in the 86-kDa β–γ band after different treatments of reduced hybrid F_1 are summarized in Fig. 4. As shown in Fig. 3 (lane 4), a substantial amount of ^{35}S appears in the 86-kDa band if catalytic turnover occurs before reoxidation. To determine the level of ^{35}S expected in the β–γ band if γC87 has an equal chance to react with all three β subunits in hybrid F_1, the control sample shown in lane 9 was run. This hybrid was reconstituted from a mixture of βD380C-F_1 and ^{35}S-labeled βD380C/γC87S-F_1 that had not been pretreated with DTNB, so that ^{35}S-labeled and unlabeled β subunits reconstituted randomly in all three positions with γC87. Oxidation of this hybrid resulted in the highest observed level of ^{35}S-labeled β subunit in the β–γ band (Fig. 3, lane 9), and this value was used to calculate the maximal expected ^{35}S in the β–γ band of hybrid F_1, assuming that all three β subunits had an equal opportunity to crosslink to γ subunit after reduction (Fig. 4, 100%). For hybrid F_1 with an initial nonradioactive crosslink, reduction followed by catalytic turnover and reoxidation (Fig. 3, lane 4) yielded 88% of the expected value (Fig. 4, MgATP). In the absence of catalytic turnover (Fig. 3, lanes 3 and 5–7), some ^{35}S was detected in the β–γ band, but this was only a fraction of that predicted for random reaction (Fig. 4, 3–25%). The absence of ^{35}S-labeled β subunit in the 86-kDa band of the nonhybrid control (Fig. 3, lane 8, in which crosslinked βD380C-F_1 and ^{35}S-labeled βD380C/γC87S-F_1 were reconstituted separately before mixing) excludes the possibility of β-subunit exchange between F_1 molecules after reassembly.

Although the technique used here cannot confirm whether rotation of γ subunit relative to the β subunits occurs in an ordered, sequential fashion at a kinetically competent rate, our results do allow several conclusions relevant to the rotary feature of the binding change mechanism. First, from the high-resolution structural model of bovine heart F_1, it is clear that the homolog of the *E. coli* γC87 residue cannot achieve equivalent interactions with each β subunit without rotation of the entire γ subunit within the core of F_1 (ref. 14; J. E. Walker, personal communication). Thus, our results demonstrate that γ subunit can rotate relative to the β subunits of F_1. Furthermore, this rotation occurs readily only when the enzyme is exposed to conditions for catalytic turnover. Finally, the proportion of ^{35}S-labeled β subunit incorporated into the β–γ crosslink after catalytic turnover is consistent with the ability of this specific region of the γ subunit to interact equivalently with each of the three β subunits during the course of the catalytic cycle, as predicted for the rotary mechanism.

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