comparably expressed (Fig. 4*c*, right). Similar results were obtained with Smad2 (data not shown). We concluded that the C-terminal domain of Smad proteins has transcriptional activity that can be unmasked by removal of the N-terminal domain.

The inability of the full-length Smad1 to stimulate transcription suggested that its activation may be regulated. Reasoning that activation of the full-length Smad1 may require a BMP signal, we costransfected this construct with BMP receptors types I and II in R-1B/L17 cells and analysed the effect of the ligand. Responsiveness of R-1B/L17 cells to BMP requires transfection of these two receptor serine/threonine kinases[12,13]. As shown in Fig. 4*d*, BMP4 addition to cells transfected with either the BMP receptor I (BMPR-IA) or receptor II (BMPR-II) did not activate GAL4–Smad1(FL). In contrast, BMP4 addition to cells cotransfected with both BMPR-IA and BMPR-II increased the transcriptional activity of GAL4–Smad1(FL) ~5-fold relative to the same cells without BMP4 (Fig. 4*d*). Similar results were obtained with BMP2 or the receptor isoform BMPR-IB (data not shown). This effect is dependent on the kinase activity of the receptors because kinase-defective mutant versions of BMPR-IA (Fig. 4*d*) or BMPR-II (data not shown) failed to mediate this BMP4 response. Controls showed that BMP4 does not increase the expression of the GAL4-Smad1(FL) protein, and cotransfection of BMPR-IA and BMPR-II, with or without BMP4 addition, does not increase the activity of GAL4(1–147) (data not shown).

In conclusion, we have shown that Smad1 is concentrated in the nucleus upon BMP stimulation and that it can mediate BMP signals. Moreover, we have shown that Smad1 and other Smad proteins, including DPC4, encode transcriptional activators. This activity is located in their C-terminal domain and is unmasked upon removal of the N-terminal domain. We further demonstrate that the transcriptional activity of Smad1 can be stimulated by BMP-receptor-mediated signals. How these signals regulate the transcriptional activity of Smad1 is not clear. We have observed that BMP stimulation increases Smad1 phosphorylation (data not shown), but it remains to be determined whether phosphorylation regulates the transcriptional activity of Smad1. The finding that Smad1 and other Smad proteins are transcriptional activators is a step towards a better understanding of gene regulation by the TGF-β family.

Note added in proof: *Smad1* is identical to the recently published *Madr1* (ref. 22) and is the homologue of *Xenopus Xmad1* (ref. 23). □

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CORRESPONDENCE and requests for materials should be addressed to J.M. (e-mail: j-massague@ski.mskcc.org).

Intersubunit rotation in active F-ATPase

D. Sabbert, S. Engelbrecht & W. Junge

Abteilung Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück, D-49069 Osnabrück, Germany

The enzyme ATP synthase, or F-ATPase, is present in the membranes of bacteria, chloroplasts and mitochondria. Its structure is bipartite, with a proton-conducting, integral membrane portion, F_o, and a peripheral portion, F_1. Solubilized F_1 is composed of five different subunits, $(\alpha\beta)_3\gamma\delta\varepsilon$, and is active as an ATPase[1,2]. The function of F-ATPase is to couple proton translocation through F_o with ATP synthesis in F_1 (ref. 3). Several lines of evidence support the spontaneous formation of ATP on F_1 (refs 4, 5) and its endergonic release[6] at cooperative and rotating (or at least alternating) sites[7]. The release of ATP at the expense of protonmotive force might involve mechanical energy transduction from F_o into F_1 by rotation of the smaller subunits (mainly γ) within $(\alpha\beta)_3$, the catalytic hexagon of F_1 as suggested by electron microscopy[8], by X-ray crystal structure analysis[9] and by the use of cleavable crosslinkers[10]. Here we record an intersubunit rotation in real time in the functional enzyme by applying polarized absorption relaxation after photobleaching to immobilized F_1 with eosin-labelled γ. We observe the rotation of γ relative to immobilized $(\alpha\beta)_3$ in a timespan of 100 ms, compatible with the rate of ATP hydrolysis by immobilized F_1. Its angular range, which is of at least 200 degrees, favours a triple-site mechanism of catalysis[7,11], with γ acting as a crankshaft in $(\alpha\beta)_3$. The rotation of γ is blocked when ATP is substituted with its non-hydrolysable analogue AMP-PNP.

We investigated intersubunit rotation in the active F_1-ATPase CF_1 from spinach chloroplast. The enzyme was purified and modified covalently by eosin-5-maleimide on subunit-γ. Figure 1 shows the specificity of labelling, as indicated by SDS-PAGE. The Labelled enzyme was immobilized on Sephadex DEAE-A50 (ref. 12). We used polarized absorption relaxation after photobleaching

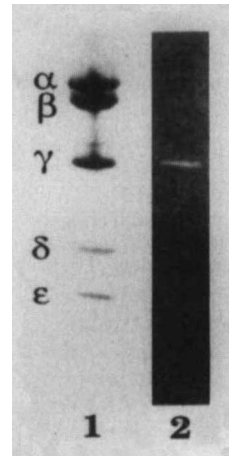

FIG. 1 Covalent modification of chloroplast F_1-ATPase, CF_1, with eosin-5-maleimide. SDS-PAGE of CF_1 (lane 1), eosin-5-maleimide labelled CF_1 (lane 2). Samples were electrophoresed in Pharmacia Phast gradient gels (8–25%), and silver-stained (lane 1)[19] or photographed under UV light (lane 2); 0.3 μg protein was loaded per lane.

METHODS. CF_1 was prepared by anion-exchange chromatography of EDTA extracts from spinach chloroplasts[20]. The specific activity of the enzyme was 25 U mg^{-1} (measured in 50 mM Tris-HCl, 5 mM ATP, 2 mM $MgCl_2$, 30 mM 1-*O*-*n*-octyl-β-D-glucopyranoside (Sigma), 10 mM Na_2SO_3, with 5 min incubation at 37 °C); the reaction was terminated with 0.5 M trichloroacetic acid. Phosphate concentration was determined according to ref. 21. Specific labelling of CF_1 (5 μM) was achieved at room temperature for 10 min in the dark at pH 7.0 (50 mM MOPS-NaOH) with 50 μM eosin-5-maleimide (Molecular Probes); the reaction was terminated with 1 mM *N*-acetylcysteine. Labelling decreased the specific Mg-ATPase activity from 25 to 20 U mg^{-1}. The reaction with eosin-5-maleimide occurred in the γ-subunit. Under these conditions, eosin-5-maleimide is expected to react only with the so-called 'dark site' in spinach chloroplast γ, namely residue CF_1-γ(C322)[16,17]. The stoichiometric proportion of bound dye (extinction coefficient at 530 nm, 10^5 M^{-1} cm^{-1}) over enzyme was >50% (mol per mol). Labelled CF_1 was immobilized on Sephadex DEAE-A50. Unbound CF_1 was removed by washing. Upon immobilization the specific activity of eosin-labelled CF_1 was decreased ~10-fold to 2 ± 1 U mg^{-1} (ATP-turnover number, 14 ± 7 s^{-1}).

(PARAP) to detect slow rotational motion of eosin-labelled subunit-γ relative to immobilized $(\alpha\beta)_3$. The results of PARAP are illustrated in Fig. 2. The decay of the polarization anisotropy parameter, r, reflects only and directly the rotational motion of the chromophore (Fig. 2, bottom)[13,14]. If the same non-degenerate optical transition is both excited and interrogated, as in our experiments with eosin, the theoretically expected initial value of r is 0.4 (ref. 14). If the chromophore rotates fully around all three cartesian axes, r decays to zero. If the rotation is more restricted (dimensionally or by boundaries), r may decay to a value greater than zero. If the linear oscillator rotates uniaxially in the plane of a circle, r decays to a value of 0.1 (ref. 15). Broadly, the rate constants of the decay of r are indicative of the 'velocity' of rotational motion, and its steady value is indicative of restrictions to full rotational freedom.

Figure 3 shows the value of the anisotropy parameter as a function of the time after firing the laser flash. CF_1 was labelled with eosin-5-maleimide at the C terminus of subunit-γ at Cys322 (refs 16,17). The initial value of the anisotropy was $r(t = 0) = 0.1$, smaller than the theoretical maximum. This was due to a very fast spinning of the chromophore around its single bonds (nanosecond timescale). r decayed to a stationary value of $r(t \rightarrow \infty) = 0.018$ (Fig. 3, middle trace). This decay was only observed under conditions of hydrolysis: that is, in the presence of ATP and $MgCl_2$ to drive the enzyme, and of *n*-octyl-β-D-glucopyranoside at 37 °C to activate it. Its time constant was of the order of 100 ms (a theoretical analysis, see fit line in Fig. 3, will be presented elsewhere). If, on the other hand, ATP was substituted by its non-hydrolysable analogue AMP-PNP, the slow decay of r was blocked (upper trace). When CF_1 was free in aqueous buffer solution, r was zero (Fig. 3, lower trace). The reduction from 0.4 to zero was due to the very rapid rotational diffusion of CF_1 in solution (rotational correlation time ~200 ns; ref. 18). Neither the librational motion of the dye, nor the rapid rotational diffusion of the protein were time-resolved in these experiments. The possibility that the activity-linked decay of the polarization anisotropy could be due to a non-immobilized subset of CF_1-molecules is eliminated by the constancy of r in the presence of AMP-PNP.

Figure 4*a* shows a space-filling model of $(\alpha\beta)_3$, with the front region removed to expose subunit-γ, displayed as a ribbon. The α-helix extending almost to the top terminates at the C terminus of γ. In the chloroplast enzyme, its penultimate residue is Cys 322, which reacts with eosin-5-maleimide, here illustrated by its transition moment as rotating on a circle (Fig. 4*b*). As already emphasized[9], the crystal structure of mitochondrial F_1ATPase leaves little freedom for the librational motion of the long axis of γ. Although in a dynamic situation, the bearing for γ in $(\alpha\beta)_3$ may be looser than suggested by the stationary crystal picture, we assume that the angle ρ in Fig. 4*b* is small and negligible (a quantitative analysis will be presented elsewhere). Two rotational degrees of freedom remain: first, rapid brownian rotation around the single linker bonds(s) of the chromophore with the rotation axis inclined at an angle Θ against the long axis of γ, and second, the rotation of γ around its long axis over an angular interval ϕ (Fig. 4*b*).

We interpreted the steady level of r observed under ATP hydrolysis, namely $r(t \rightarrow \infty) = 0.018$, in terms of Wahl's[15] theory of angularly restricted, uniaxial brownian rotation. Adapting his equation (50) to our case, we obtained an expression linking the observed value of $r(t \rightarrow \infty)$ with the two angles Θ and ϕ (Fig. 4*c* legend). A contour plot of $r(t \rightarrow \infty, \theta, \phi)$ over the

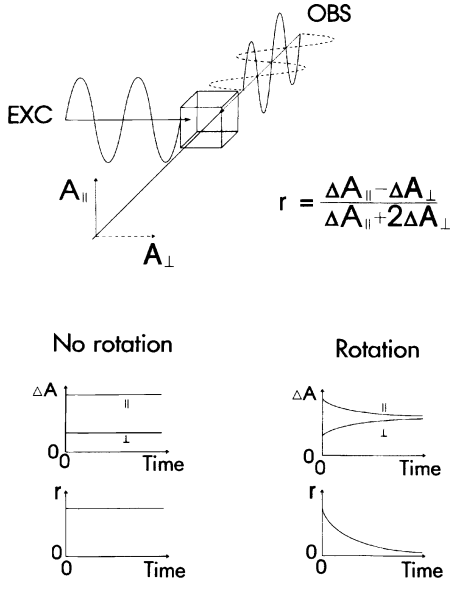

FIG. 2 Polarized absorption relaxation after photobleaching (PARAP). Two beams of linearly polarized light, the laser flash and the measuring beam, impinge at right angles on the cuvette containing an originally isotropic sample of labelled CF_1. The exciting laser flash causes absorption transients which are recorded by the continuous measurement light.
METHODS. The sample was excited by a linearly polarized laser flash (Q-switched Nd-YAG at 532 nm for 5 ns) at non-saturating energy (0.4 mJ mm^{-2}). The flash excites an oriented subset of chromophore molecules with more or less parallel orientation of their transition moments to the ***E*** vector of the laser light. With about 70% probability, eosin crosses over from its excited singlet state into the triplet state[22] and decays into the electronic ground state in <1 ms. Only a minority of molecules (about 0.2% in the absence of oxygen) is irreversibly bleached. The bleaching of this minority is used to probe the putative slow rotation of γ. We measured absorption transients at 520 nm, attributable to the same $\pi \rightarrow \pi*$ transition that is excited by the laser flash at 532 nm. Because of the orientational photoselection by the linearly polarized flash, the extent of absorption transients, which are detected by a linearly polarized, continuous measuring light, differs according to whether the ***E*** vector of the interrogating beam is polarized in parallel ($\Delta A_{\parallel}$) or perpendicularly ($\Delta A_{\perp}$) to the one of the exciting flash. If the chromophores are fixed, the difference is constant. It decays if they rotate, as illustrated in the lower portion of the figure. Usually rotational motion is analysed in terms of the decay of the anisotropy parameter, r (ref. 14): $r(t) = \Delta A_{\parallel} - \Delta A_{\perp} / \Delta A_{\parallel} + 2\Delta A_{\perp}$. If the same non-degenerate optical transition is both excited and interrogated, as in our experiments with eosin, the theoretically expected initial figure of r is 0.4 (ref. 14). With immobilized eosin in polyacrylamide we observed a value of 0.37 in the set-up shown in Fig. 2, conforming with this expectation.

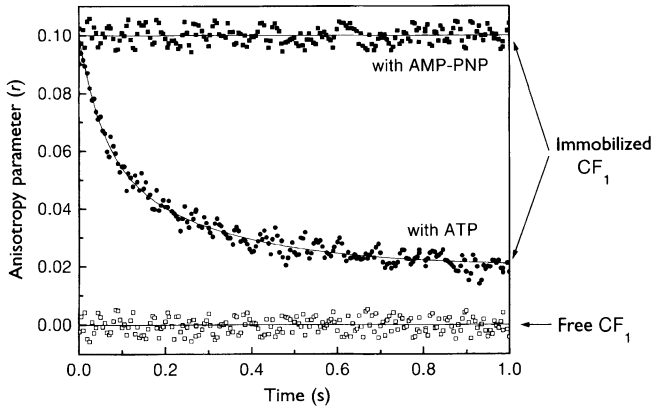

FIG. 3 The anisotropy parameter r as function of the time after a laser flash at time zero to an isotropic ensemble of eosin-labelled CF_1. The sample, 0.5 mg CF_1 immobilized per ml Sephadex DEAE-A50 (suspended in Tris-HCl, pH 7.8) in a quartz cuvette ($5 \times 5 \times 5\ mm^3$), was treated with 2 mM $MgCl_2$, 30 mM *n*-octyl-β-D-glucopyranoside and 5 mM ATP. Addition of β-D(+)-glucose and glucose oxidase/catalase (10/10 μg; Sigma), rendered the sample anaerobic, as verified by the phosphorescence lifetime of eosin. After 15 min incubation, the cuvette was heated to 37 °C to stimulate ATP hydrolysis. The sample was excited by repetitive laser flashes at non-saturating energy (density, 0.4 mJ mm^{-2}). Polarized absorption transients were recorded and averaged digitally. A new sample was introduced after 20% irreversible bleaching. Data points are the average of 2,000 observations. CF_1 was labelled on subunit-γ by eosin-5-maleimide (Fig. 2, lane 3). Top trace: immobilized CF_1 with 5 mM 5-adenylylimidodiphosphate (AMP-PNP); middle immobilized CF_1 with ATP; bottom: CF_1 free in solution with ATP added.

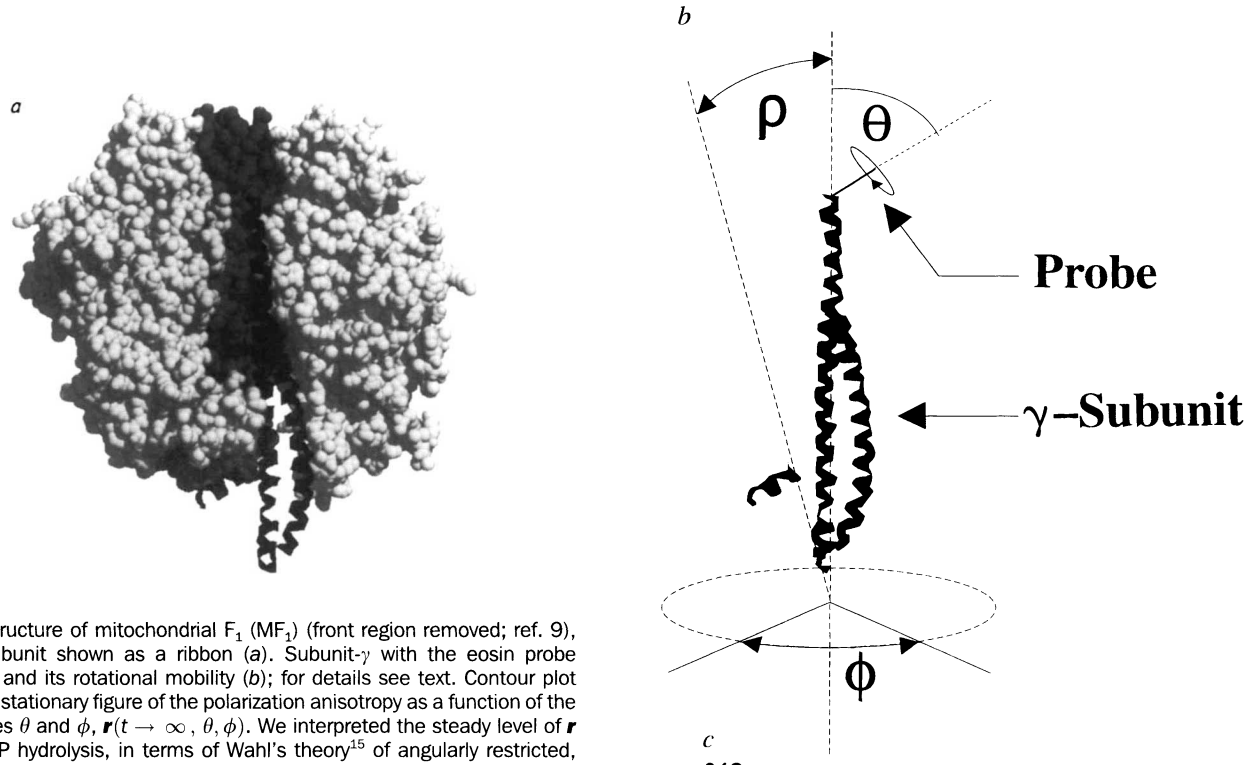

FIG. 4 Structure of mitochondrial F_1 (MF_1) (front region removed; ref. 9), with γ-subunit shown as a ribbon (*a*). Subunit-γ with the eosin probe attached and its rotational mobility (*b*); for details see text. Contour plot (c) of the stationary figure of the polarization anisotropy as a function of the two angles θ and ϕ, $\boldsymbol{r}(t \rightarrow \infty, \theta, \phi)$. We interpreted the steady level of $\boldsymbol{r}$ under ATP hydrolysis, in terms of Wahl's theory[15] of angularly restricted, uniaxial brownian rotation. Adapting his equation (50) to our case, we obtain the following expression for the extent of the steady level of $\boldsymbol{r}$:

$$\boldsymbol{r}(t \rightarrow \infty, \Theta, \phi) = \boldsymbol{r}(t=0) \cdot \left[K_1 \frac{\sin^2(\phi/2)}{(\phi/2)^2} + K_2 \frac{\sin^2\phi}{\phi^2} + K_3 \right]$$

with $K_1 = 3\sin^2\theta\cos^2\theta$, $K_2 = 0.75\sin^4\theta$, $K_3 = 0.25(3\cos^2\theta - 1)^2$, $\boldsymbol{r}(t=0) = 0.1$. The angular domain of the uniaxial rotation, ϕ, and the inclination of the binding axis of the label to the long axis of γ, θ, both of which enter into these equations, are shown in *b*. The thick line in c denotes all sets of ϕ and θ that are compatible with the experimental value of $\boldsymbol{r} = 0.018$ (hydrolysing enzyme), as obtained for the rotation of γ relative to immobilized $(\alpha\beta)_3$.

plane spanned out by Θ and ϕ is shown in Fig. 4*c*. It shows that the observed steady value of 0.018 implied at least 200 degrees (for $\theta = 60°$) of rotational freedom of γ around its long axis (angle ϕ). This was a lower limit for two reasons. First, if θ was different from 60°, the rotational freedom (angle ϕ) of γ was larger; and second, a subset of enzymes where γ was also immobilized would have increased the apparent value of $\boldsymbol{r}(t \rightarrow \infty)$ over the true value of the complement set of active enzyme molecules.

In conclusion, the hydrolysis of ATP by the chloroplast ATPase CF_1 is accompanied by the rotational motion of subunit-γ around its long axis within and relative to the hexagonal array of $(\alpha\beta)_3$, which contains the three catalytic nucleotide-binding sites. To our knowledge this is the first time that intersubunit rotation over a large angular domain has been resolved in real-time. Its rate conformed with the rate of the catalytic turnover, and its angular range extended over at least 200 degrees. These observations corroborate the functional relevance of intersubunit rotation in ATP hydrolysis. How proton translocation through the membrane portion of ATP synthase, F_o, generates the torque to rotate γ within $(\alpha\beta)_3$ of F_1 remains to be established. □

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CORRESPONDENCE and requests for materials should be addressed to W.J. (e-mail: junge@uni-osnabruck.de).