

The binding of eosin-labeled subunit δ to the isolated chloroplast ATPase, CF₁, as revealed by rotational diffusion in solution

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We investigated the binding of subunit δ to solubilized chloroplast ATPase. Purified δ was covalently labeled with eosin 5-isothiocyanate and its rotational correlation time was determined by a photoselection technique as a function of added CF₁ (containing δ) and of CF₁($-\delta$) (lacking δ). In aqueous buffer the rotational correlation time of labeled δ was 33 ns. This is compatible with a rather elongated shape with the dimensions $2b=100 \text{ \AA}/2a=28 \text{ \AA}$. Binding of δ to CF₁ decreased the rotational correlation time about 10-fold. The result was a biphasic decay of the laser flash-induced absorption anisotropy which was analyzed to yield the proportion of δ (bound to CF₁) relative to δ (free). CF₁($-\delta$), which completely lacked the δ -subunit, bound one δ (mol/mol) with high affinity ($K_d \approx 100 \text{ nM}$) and at least another δ with about 20-fold lower affinity. The δ -containing CF₁ revealed only the low-affinity site(s) for δ . This was compatible with a 1:1 stoichiometry of δ in isolated CF₁.

Photophosphorylation; ATPase; Coupling factor; δ -Subunit; Rotational diffusion

1. INTRODUCTION

ATP synthesis in green plants is catalyzed by CF₀CF₁ which consists of two separate moieties: a hydrophobic membrane-spanning portion, CF₀, acting as a proton channel and a peripheral portion, CF₁, carrying the nucleotide-binding sites. Proton-translocating ATPases of the F₀F₁ type are found in mitochondria, bacteria and chloroplasts [1–3]. CF₁ is composed of five polypeptides, designated α , β , γ , δ and ϵ in order of decreasing molecular mass. The stoichiometry of CF₁ subunits has been a matter of controversy [4,5]. $\alpha_3\beta_3\gamma\delta\epsilon$ is now widely accepted [4,5]. The β -subunit contains the catalytic sites [6], whereas α has been proposed to be involved in the regulation

of catalysis [7]. The γ -subunit may be involved in proton translocation as well as in the redox-mediated conversion of CF₁ from the latent into the active state [8]. The ϵ polypeptide is involved in the regulation of ATPase activity [9]. The δ -subunit is required for the functional reconstitution of photophosphorylation in CF₁-depleted thylakoids [10–13], i.e. by its ability to block proton conduction by CF₀ [12,16,39]. Recently, it has been proposed that the stoichiometric proportion of δ in CF₀CF₁ is higher than 1 [13].

We investigated binding of the δ -subunit to soluble CF₁ via the hydrodynamic behavior of δ in solution. Using a photoselection technique we measured the rotational diffusion of eosin-labeled δ . δ was covalently labeled with eosin-NCS without loss of its reconstitutive activity. A solution of eosin-labeled δ , and with varying amounts of CF₁ present, was excited with a linearly polarized laser flash and the ground-state depletion of eosin was probed with linearly polarized light. The decay of the absorption anisotropy was multiphasic. The slow phase (decay time about 300 ns) was attributable to those molecules of δ which were

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Abbreviations: CF₁ (CF₀), soluble (membrane) portion of the chloroplast ATP synthase; eosin-NCS, eosin 5-isothiocyanate; OSCP, oligomycin sensitivity-conferring protein of the mitochondrial coupling factor; PMS, phenazine methosulfate

bound to CF₁, and one of the fast phases (about 30 ns) to those remaining which were still free in solution. Further decay components (e.g. 80 ns) were attributable to librational motion of bound dye relative to the δ-CF₁ complex. An analysis of the multiphasic decay for the equilibrium concentrations of δ, free and bound, yielded the number of binding sites and their dissociation constants.

2. MATERIALS AND METHODS

CF₁ was prepared on a large scale from spinach thylakoids as in [14–16]. Further purification of CF₁ and preparation of CF₁(-δ) and δ-subunit were performed as described [39]. For labeling of the isolated δ-subunit, the protein (0.23 mg/ml, total volume 2 ml in 25 mM Tris-HCl, pH 8.0) was incubated for 20 min with 400 μM eosin-NCS. Eosin-NCS was purchased from Molecular Probes (Eugene, OR). The stock solution (2 mg/ml eosin-NCS in 50 mM Tris-HCl, pH 8.0) was freshly prepared in order to avoid hydrolysis of the reactive -NCS group. After labeling the protein was passed twice through a prepacked PD-10 gel filtration column (Pharmacia). The protein concentration and the amount of eosin-NCS bound to protein were spectrophotometrically determined as in [17,18]. The labeled δ-subunit contained 1.05 mol eosin-NCS/mol δ. Partial depletion of thylakoids from CF₁ and reconstitution were carried out as described [12]. Preparation, labeling of the δ-subunit, reconstitution and measurement of rotational diffusion were performed on the same day. Eosin was excited by a pulse from a frequency-doubled Nd-YAG laser (534 nm, 10 ns duration, 10 mJ) and absorption changes of eosin (ground-state depletion) were monitored at a wavelength of 502 nm. The detection system (photodiode plus amplifier) was blindfolded for 30 ns by flash burst artefacts, which, however, did not produce artefacts of linear dichroism from 70 ns onwards. This was checked with totally immobilized eosin. The principles and the apparatus for measuring the rotational diffusion of macromolecules with extrinsic probes by photoselection are detailed elsewhere [18,19]. From the dichroic absorption changes of protein-bound eosin-NCS the time course of the absorption anisotropy $r(t)$ was calculated according to:

$$r(t) = (A_p(t) - A_v(t)) / (A_p(t) + 2A_v(t)) \quad (1)$$

where $A_p(t)$ and $A_v(t)$ denote the absorption changes obtained for parallel and perpendicular orientation between the polarization of the exciting laser flash and the measuring light. The decay of the experimentally determined absorption anisotropy was analyzed for exponentials by the program of Provencher, which is based on the Fourier convolution theorem. The program [20] fits noisy raw data by multiexponential decay curves, without initial guesses concerning the number of components or their decay constants being required. The functional dependence of the rotational correlation times on the three principal diffusion coefficients of arbitrarily shaped macromolecules in isotropic solution are known from the literature [21]. These coefficients provide information on the hydrodynamic shape [22,23].

3. RESULTS AND DISCUSSION

3.1. Preparation of CF₁ and δ-subunit

The δ-subunit and CF₁(-δ) were prepared from CF₁ according to [39]. Both samples were pure as judged from silver-stained gels on SDS electrophoresis (fig.1). Lane c shows the starting material CF₁, lane b the purified δ-subunit and lane a CF₁(-δ). After labeling with eosin-NCS the δ-subunit contained 1.05 mol eosin-NCS per mol.

In order to control the functional integrity of the eosin-labeled δ-subunit and the CF₁(-δ) preparation, CF₁(-δ) was added to CF₁-depleted thylakoids in the presence of unlabeled and labeled δ-subunit, respectively (for experimental details see [12,39]). The CF₁-depleted thylakoids retained 42% control activity in PMS-mediated ATP synthesis. Addition of CF₁(-δ) enhanced the rate of photophosphorylation to 67%. Addition of CF₁(-δ) together with the δ-subunit resulted in a further enhancement in the rate of photophosphorylation to 77% (unlabeled δ) and 73% (eosin-labeled δ). This demonstrated that the preparations of CF₁(-δ) and δ used in laser flash spectrophotometry recombined to physiologically competent CF₁ and that labeling of the δ-subunit with eosin-NCS did not impair this capability.

3.2. Rotational diffusion of the eosin-NCS labeled δ-subunit

Fig.2 shows the time course of the absorption anisotropy calculated (point by point) from the absorption transients for parallel and perpendicular polarization according to eqn 1. Analysis of this particular measurement revealed that the absorption anisotropy decayed monoexponentially according to $r(t) = 0.17\exp(t/33 \text{ ns})$. The expected rotational correlation time of a spherical molecule with a δ-subunit molecular mass of 21 kDa can be calculated according to:

$$t_{\text{sph}} = V_{\text{hyd}}\eta/kT \quad (2)$$

where V_{hyd} is the hydrated volume of the macromolecule, k Boltzmann's constant and η the viscosity of the medium. The hydrated volume may be estimated from the relationship:

$$V_{\text{hyd}} = M_r(v_p + v_1h)/N_A \quad (3)$$

where v_p and v_1 denote the partial specific volumes of the protein and the solvent, respectively, and N_A

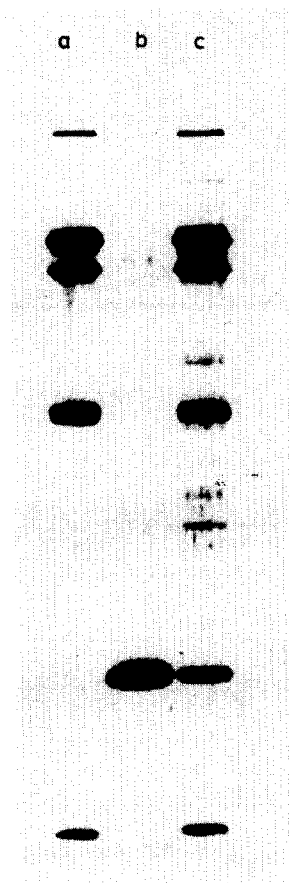


Fig.1. SDS electrophoresis with 12% separating and 5% stacking gels according to Laemmli [26] and Mattick et al. [27]; 11 mA, 16 h. Migration from top (-) to bottom (+). Silver staining was performed as in [14]. Lane c shows the CF_1 preparation from which δ was prepared (lane b) and $CF_1(-\delta)$ (lane a) according to [14].

is Avogadro's number. With $v_p = 0.745$ and $h = 0.5$ g water/g protein, as is common for globular proteins [22], we obtained $V_{hyd} = 4.34 \times 10^{-20}$ cm³ and $t_{sph} = 10.7$ ns. The difference between the expected rotational correlation time for a normally hydrated spherical protein of the same molecular mass as the δ -subunit and the value observed experimentally indicated that δ was a highly hydrated protein and/or its shape strongly deviated from being spherical. Assuming the usual level of hydration and approximating δ by a prolate ellipsoid of revolution, we calculated [22,23] an axial ratio of at least $a/b = 1:3.6$. The diameter of the longer

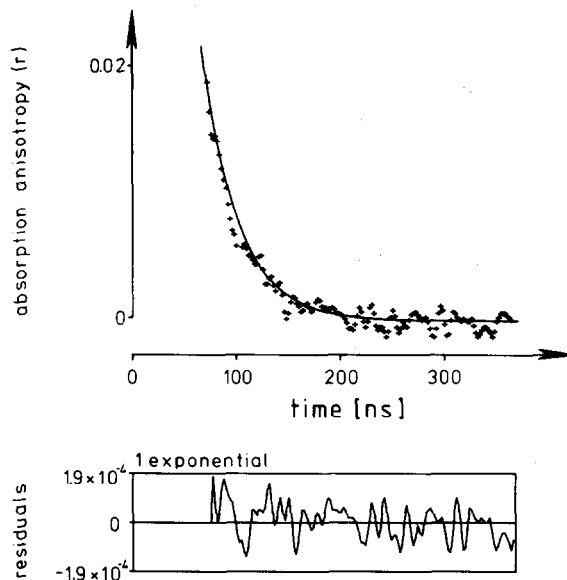


Fig.2. Time course of the absorption anisotropy of eosin-NCS labeled δ -subunit. The sample (final volume 2 ml) contained 6.6 μ M eosin and 0.132 mg/ml protein in 25 mM Tris-HCl, pH 8.0. The inserted solid line shows the fit of the data by exponential decay curves (see section 2), which gave a standard deviation of 6.4×10^{-4} . The corresponding residuals of the fit are shown at the bottom. The time course shown was calculated according to eqn 1 from the absorption changes $A_{\parallel}(t)$ and $A_{\perp}(t)$ obtained for parallel and perpendicular polarization between measuring light and excitation flash averaged over 200 single events each.

axis of δ was $2b = 10$ nm and that of the shorter axis $2a = 2.8$ nm. Oblate geometry implied an even greater axial ratio of 8:1, which made an oblate structure of the δ -subunit unlikely. The inferred prolate shape was in fair agreement with the results of Schmidt and Paradies [31], who proposed that the δ -subunit is a prolate ellipsoid with $2a = 2.5$ nm, $2b = 2.8$ nm and $2c = 9$ nm, based on sedimentation velocity and X-ray small-angle scattering data.

3.3. Equilibrium binding of the eosin-NCS labeled δ -subunit to $CF_1(-\delta)$

Binding of the labeled δ -subunit to $CF_1(-\delta)$ was followed by measuring the rotational diffusion of the δ -subunit in the presence of increasing concentrations of $CF_1(-\delta)$. The time interval between addition of $CF_1(-\delta)$ aliquots to labeled δ -subunit and measurement was 20 min. The time course of the absorption anisotropy of eosin-labeled δ in the

presence of $CF_1(-\delta)$ (trace b, molar ratio $1 \delta/2 CF_1(-\delta)$) is shown in fig.3. For comparison the absorption anisotropy of δ in the absence of $CF_1(-\delta)$ is shown as trace a (same as in fig.1). The absorption anisotropy for trace b decays more slowly than that for trace a. For trace b the analysis yielded:

$$r(t) = 0.09\exp(-t/37 \text{ ns}) + 0.065\exp(-t/290 \text{ ns})$$

in contrast with that for trace a:

$$r(t) = 0.17\exp(-t/33 \text{ ns}).$$

Decreasing the ratio of δ to $CF_1(-\delta)$ increased the extent of the slower component, however its relaxation time remained constant (300 ± 10 ns). In contrast, the extent and the relaxation times of the fast component varied considerably. The relaxation time of the fast component increased from 33 to about 80 ns at $CF_1(-\delta)/\delta$ molar ratios above 2.

The rotational correlation time of about 300 ns was attributed to rotational diffusion of labeled δ -subunit when bound to CF_1 . This value falls within the known range for latent CF_1 [18]. The shorter correlation time may result from the unresolved mixing of both rotational diffusion of free δ and restriction motion of the label on δ bound to CF_1 [18,19,33,34]. Since the values of these two components were both in the range of the flash burst artefact of our instrument, further discrimination was not possible. We took the extent of the slowly relaxing component as a measure of the amount of δ which was bound to CF_1 . In order to determine its upper limit we increased the molar ratio of $CF_1(-\delta)$ to δ to values where the magnitude of the slower component was not further increased.

The result of such an experiment is listed in table 1. Table 1 clearly demonstrates that for increasing $CF_1(-\delta)$ concentrations the extent of the slower component approached saturation at a value of $r_2(0) \approx 0.07$ and concomitantly the relaxation time of the faster component increased to 80 ± 20 ns. Even at molar ratios of 10:1 between $CF_1(-\delta)$ and δ (not shown), no further increase in the extent of the slower component was observed. We therefore concluded that the new fast component (relaxation time 80 ns) which appeared at high $CF_1(-\delta)/\delta$ ratios was due mainly to segmental motion rather than to rotational diffusion of free δ . Since the same results were obtained with 3 different preparations of δ , we tentatively excluded the

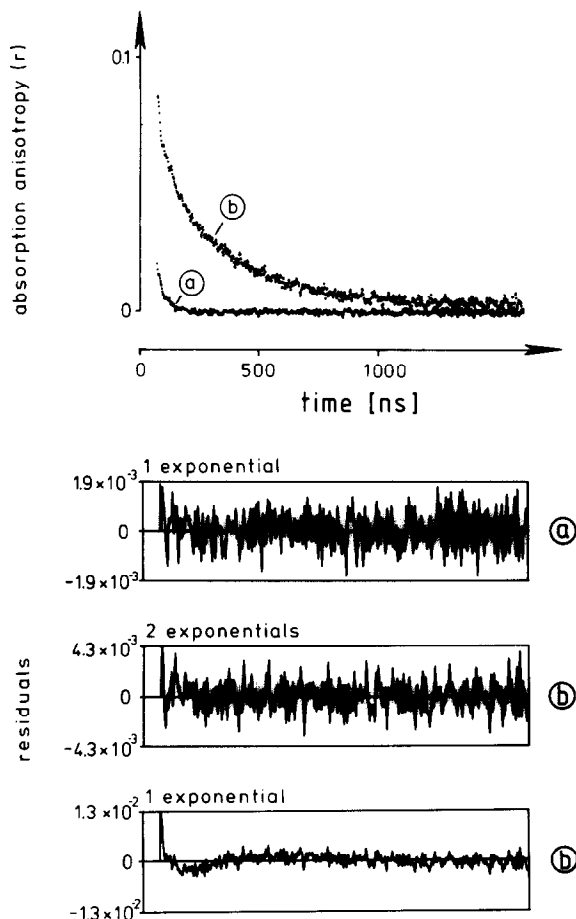


Fig.3. Time course of the absorption anisotropy of eosin-NCS labeled δ -subunit in the absence (a) and presence of $CF_1(-\delta)$ (b). The sample in trace (a) was essentially the same as that described in the legend to fig.2. In trace (b) the sample (final volume 3.2 ml) contained $60 \mu\text{g } \delta/\text{ml}$ and $2.3 \text{ mg } CF_1(-\delta)$; the concentration of eosin-NCS bound to δ was $3 \mu\text{M}$. For relaxation times and amplitude components see text. The fit of trace (b) gave a standard deviation of 1.26×10^{-3} (one exponential) and 6.4×10^{-4} (two exponentials). The corresponding residuals of the fits for traces (a,b) are shown at the bottom.

possibility of structural inhomogeneity of the δ preparations.

We used the extent of the slowly relaxing component of the absorption anisotropy to calculate the binding of δ to $CF_1(-\delta)$. At saturation, $r_2(0) \approx 0.07$, all of the labeled δ -subunit was assumed to be bound to $CF_1(-\delta)$.

As shown by Weber et al. [28], the observed absorption anisotropy in a polarization- or rotation-

Table 1

Initial extent of the absorption anisotropy, slowly relaxing component, $r_{\text{slow}}(0)$, as a function of the total concentration of δ and of $\text{CF}_1(\delta)$

| | $[\delta]_0$ (μM) | $[\text{CF}_1(-\delta)]_0$ (μM) | $r_{\text{slow}}(0)$ |
|----|-----------------------------------|---|----------------------|
| 1 | 4.3 | 0.0 | 0.0 |
| 2 | 3.9 | 0.8 | 0.023 |
| 3 | 3.5 | 1.5 | 0.041 |
| 4 | 3.2 | 2.1 | 0.043 |
| 5 | 3.0 | 2.5 | 0.050 |
| 6 | 2.8 | 2.9 | 0.056 |
| 7 | 2.6 | 3.2 | 0.054 |
| 8 | 2.2 | 3.8 | 0.064 |
| 9 | 2.0 | 4.2 | 0.065 |
| 10 | 1.7 | 4.7 | 0.066 |

wise inhomogeneous sample is additive in terms of the anisotropies of the subsets:

$$r(t)_{\text{obs}} = \sum f_i r_i(t) \quad (4)$$

where the weight factors are given by:

$$f_i = (A_{pi} + 2A_{vi}) / \sum (A_{pk} + 2A_{vk}). \quad (5)$$

Assuming that the extinction coefficient for eosin was unchanged upon binding of eosin-labeled δ to CF_1 , the f_i were proportional to the relative concentration of free δ and of δ which was bound to CF_1 .

From the data in table 1 the concentration of bound δ could therefore be calculated according to:

$$[\delta]_b = [\delta]_0 r(0)_{\text{slow}} / 0.07 \quad (6)$$

where $[\delta]_0$ is the total δ concentration and $r(0)_{\text{slow}}$ the initial extent of the slowly relaxing component. The factor 0.07 was the maximal anisotropy of the slower component when all δ was bound to CF_1 .

Fig. 4a shows a Scatchard plot of the data in table 1. The amount of bound δ -subunit was calculated according to eqn 6. This plot revealed a biphasic course typically observed when two classes of independent binding sites are involved. One abscissa intercept was extrapolated to a binding stoichiometry of $n_1 = 1$ and the other, less well defined, to values of $n_1 + n_2 \approx 2$ or even higher. This indicated that one molecule of $\text{CF}_1(-\delta)$ bound one molecule of δ -subunit with high affinity and at least one further copy with

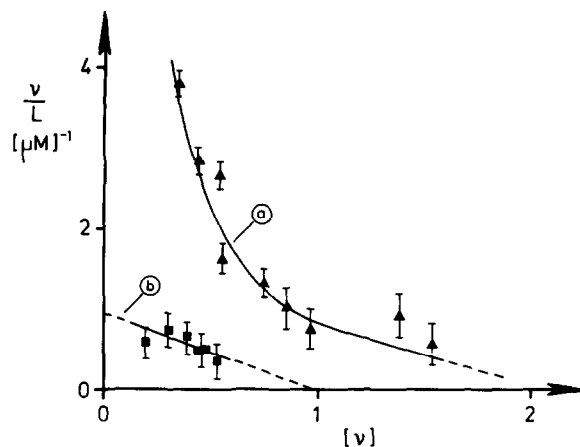


Fig. 4. Scatchard plot of the data from tables 1,2. For details see text.

lower affinity. The Scatchard plot was further analyzed according to [35]:

$$v/[L] = \sum ((n_i/K_i) / (1 + [L]/K_i)) \quad (7)$$

where $v = [\delta]_b / \text{CF}_1(-\delta)_0$, $[L] = [\delta]_f$, $K_i =$ dissociation constant, and $n_i =$ multiplicity of binding site i . This yielded a dissociation constant of $K_1 \approx 100 \pm 50$ nM for the high-affinity site with $n_1 = 1$ and, less well-defined, $K_2 \approx 2 \mu\text{M}$ for the low-affinity site(s). Soluble $\text{CF}_1(-\delta)$ contained at least two binding sites for the δ -subunit which greatly differed in affinity.

We expected that standard techniques for the solubilisation and purification of δ -containing CF_1 from thylakoid membranes should result in CF_1 containing only one copy of the δ -subunit bound to the high-affinity site. We investigated whether δ -containing CF_1 could bind at least a second copy of δ at low-affinity sites. To unlabeled CF_1 which already contained approx. 1 copy of δ as judged by SDS gel electrophoresis 20% (mol/mol) unlabeled δ was added in order to saturate residual free high-affinity binding sites. This solution was combined with labeled δ to yield an approx. 9-fold molar excess of CF_1 over labeled δ . The absorption anisotropy of this sample decayed biphasically with a time constant of ≈ 80 ns for the faster component and ≈ 300 ns for the slower component, the latter with an amplitude of $r_2(0) = 0.07$. The results are listed in table 2 and shown as a Scatchard plot in fig. 4b.

In order to evaluate whether the observed

Table 2

Initial extent of the absorption anisotropy, slowly relaxing component, $r_{\text{slow}}(0)$, as a function of the total concentration of δ and of δ -containing CF_1

| | $[\delta]_0$ (μM) | $[\text{CF}_1(-\delta)]_0$ (μM) | $r_{\text{slow}}(0)$ |
|---|-----------------------------------|---|----------------------|
| 1 | 2.7 | 11.8 | 0.063 |
| 2 | 3.7 | 10.8 | 0.064 |
| 3 | 4.5 | 10.0 | 0.062 |
| 4 | 5.3 | 9.3 | 0.059 |
| 5 | 6.0 | 8.6 | 0.055 |
| 6 | 2.5 | 3.5 | 0.046 |

The molecular mass values used to calculate the molar concentrations were 21 kDa (δ) and 389 kDa ($\text{CF}_1(-\delta)$); for further details see text

binding of the labeled δ -subunit was reversible we diluted one sample 2.5-fold with buffer and again measured the time course of the absorption anisotropy. This result is listed in the final row of table 2. Linear extrapolation of the data in fig.4b yielded an intercept on the abscissa indicative of $\nu = n \approx 1$ and another on the ordinate pointing to a dissociation constant of about $2 \mu\text{M}$, both with large error. The values obtained after dilution of the sample showed that the binding of δ to low-affinity site(s) was reversible.

We investigated whether binding of CF_1 (low affinity) was paralleled by the binding of another protein of the same size as δ . For this purpose, eosin-labeled soybean trypsin inhibitor (21 kDa) was mixed with CF_1 . Covering the same protein concentration range as given in table 2, we observed only a rapid relaxation of the absorption anisotropy (< 30 ns). This excluded nonspecific absorption of at least this polypeptide to CF_1 . Similarly, addition of eosin alone to CF_1 in the appropriate concentration range did not produce linear dichroism in the time domain discussed which excluded nonspecific absorption of the eosin moiety in δ to CF_1 .

In conclusion, we found a single high-affinity binding site for subunit δ on isolated $\text{CF}_1(-\delta)$ in solution. The dissociation constant, $K_d = 100 \pm 50$ nM, was in the same range as that for OSCP, the δ analogue in mitochondria, to MF_1 [36]. The high-affinity binding site was virtually absent (since saturated) in δ -containing CF_1 . Both CF_1 and $\text{CF}_1(-\delta)$ contained at least one additional

binding site with about 20-fold lower affinity ($K_d \approx 2 \mu\text{M}$). This also paralleled OSCP binding to MF_1 [36,37].

Our results support the widely assumed stoichiometry of 1 mol δ per mol CF_1 which coincides with the respective stoichiometry in the corresponding enzymes of *E. coli* and the thermophilic bacterium PS3 [4,40]. Our measurements were carried out with the isolated ATPase, CF_1 , where, after isolation, only the high-affinity site was occupied by δ . One may speculate about the role in the intact ATP synthase, CF_0CF_1 , of the low-affinity site(s), which was (were) also apparent in isolated CF_1 . Two alternatives are conceivable: (i) CF_0CF_1 contains more than one copy of δ [13]. In this case, our results indicate an affinity decrease for δ of certain sites on the $\alpha_3\beta_3\gamma$ moiety after isolation of CF_1 ; (ii) CF_0CF_1 contains only one copy of δ . This is supported by our observation that spinach thylakoids contained $0.02 \pm 10 \mu\text{g } \delta$ ($n = 4$) and $0.45 \pm 10 \mu\text{g } \text{CF}_1$ per μg chlorophyll ($n = 5$) (determined by immunoassay; Engelbrecht, S., unpublished). It is then conceivable that the single copy rotates between three possible contact sites for δ on the $\alpha_3\beta_3\gamma$ moiety as is compatible with the postulated alternating (or rotating) site mechanism for ATP synthesis [38].

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