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THE USE OF AUROVERTIN TO DETERMINE THE F_1 CONTENT OF SUBMITOCHONDRIAL PARTICLES AND THE ATPase COMPLEX

JAN A. BERDEN and GERRIT J. VERSCHOOR

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)

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Summary

(1) The concentration of aurovertin-binding sites calculated from fluorimetric titrations of submitochondrial particles is equal to the F_1 concentration, calculated from the concentration of F_1-binding sites in stripped particles.

(2) Direct binding experiments show that the fluorescence enhancement of aurovertin bound to submitochondrial particles and the isolated ATPase complex is less (or absent) at higher concentrations than at lower concentrations. The binding data can be described by 'specific' and 'non-specific' binding. The concentration of the 'specific' sites is twice that derived from fluorimetric titrations.

(3) After dissociation of the bound F_1 with LiCl, fluorimetric titrations with aurovertin yield linear Scatchard plots. The fluorescence enhancement and K_D are equal to those of the β-subunit-aurovertin complex. The concentration of β-subunits is double the concentration of F_1.

(4) It is concluded that both for submitochondrial particles and the isolated ATPase complex the most reliable and simple way to determine the F_1 content is to dissociate the F_1 with LiCl, spin down the insoluble material and titrate the supernatant (containing free β-subunit) with aurovertin.

Introduction

Measurements based on the specific enhancement of the fluorescence of aurovertin upon binding to mitochondrial coupling factor F_1 have shown that this protein contains two equivalent binding sites [1,2]. Sub-mitochondrial particles [3] and the ATPase complex [4] also bind aurovertin with a large enhancement of the fluorescence, but the concentration of aurovertin-binding sites calculated from fluorimetric titrations is equal to the concentration of F_1, calculated from the concentration of F_1-binding sites in F_1-stripped prepara-

tions [4,5] and not to double the F_1 concentration. This apparent discrepancy is examined in this paper.

Materials and Methods

Submitochondrial particles were prepared from heavy bovine-heart mitochondria [6] according to published procedures: A particles according to Fessenden and Racker [7], T particles according to Kagawa and Racker [8] and sucrose particles by sonicating the mitochondria continuously for 4 min (temperature rises to 30°C) in 0.25 M sucrose, removing the unbroken mitochondria by centrifugation at 10 000 × *g* and collecting the particles by centrifugation at 15 000 × *g* for 30 min. The ATPase complex was prepared as described earlier [4]. LiCl treatment of both submitochondrial particles and ATPase complex was carried out in the absence of ATP, as described for F_1 [9]. F_1 was prepared by the method of Knowles and Penefsky [10].

Aurovertin was prepared in this laboratory by Dr. Bertina [11]. Its concentration was determined by measuring the absorbance at 367.5 nm (A = 29 $mM^{-1} \cdot cm^{-1}$, [2]). Antimycin was obtained from Sigma. Its concentration in ethanol was determined spectrophotometrically at 320 nm (A = 4.8 $mM^{-1} \cdot cm^{-1}$, [12].

The fluorescence experiments were carried out using an Eppendorf fluorimeter, primary filter 313 + 366 nm and secondary filter 420—3000 nm. For the Oxygraph experiments a Clark oxygen electrode was used.

Results

Binding of aurovertin to particles and ATPase complex as measured fluorimetrically. Titrations of A particles with aurovertin yielded results very similar to those of van der Stadt et al. [3]. The fluorescence enhancement upon binding was 120-fold, the same as with isolated F_1, and the Scatchard plots were non-linear. The concentration of binding sites was usually about 0.4 nmol/mg protein. For the representation of the experimental data shown in Fig. 1, we did not use the Scatchard representation (([bound]/[free]) vs. [bound]), but, as suggested by Muller et al. [2], have plotted ([bound]/[free]) vs. (1/[bound]). This way of plotting usually yielded straight lines, improving the accuracy of the extrapolation to [bound]/[free] = 0. Since A particles have lost part of the original F_1 content, we also measured the binding of aurovertin to T particles and sucrose particles. For both types of particles the concentration of aurovertin-binding sites was about 0.55 nmol/mg protein, which is also the F_1 concentration for T particles [3].

As already reported [4], similar results were obtained with the ATPase complex: the Scatchard plots were non-linear, but plotting ([bound]/[free]) vs. (1/[bound]) yielded straight lines, from which the concentration of aurovertin-binding sites could be calculated to be 1.5 nmol/mg protein, which is about the same as the expected F_1 concentration. It appears then, that with both submitochondrial particles and ATPase complex the fluorimetric titrations yield a concentration of aurovertin-binding sites equal to the F_1 concentration.

Direct binding experiments. The binding of aurovertin to submitochondrial

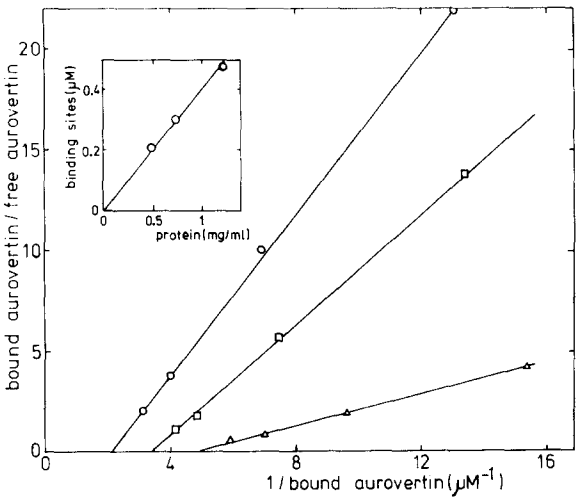

Fig. 1. Fluorimetric titration of A particles with aurovertin. Three different concentrations of A particles in 0.25 M sucrose/10 mM Tris-HCl buffer (pH 8.0) were titrated with aurovertin, but because of the long time needed for equilibrium after each addition, only four additions were made. The fluorescence of bound aurovertin was determined by extrapolation to infinite protein concentration. The enhancement of fluorescence upon binding was 125-fold. [Bound aurovertin]/[free aurovertin] is plotted against inverse of [bound aurovertin] (see ref. 2). △, 0.49 mg protein/ml; □, 0.74 mg protein/ml; ○, 1.23 mg protein/ml. The inset shows the concentration of binding sites (derived from the main figure) for the three protein concentrations used. The slope equals 0.4 nmol binding sites/mg protein

particles was also measured directly by adding different amounts of aurovertin to a particle suspension and, after centrifugation, determining the amount of aurovertin in the supernatant by measuring the fluorescence of a sample in the presence of excess F_1. The concentration of F_1 was so high that all the aurovertin added was bound, and the calibration curve with known amounts of aurovertin gave a linear relationship between aurovertin added and the measured fluorescence. Knowing the free aurovertin concentration the concentration of bound aurovertin could be calculated. An example for the binding of aurovertin to A particles (30 min was allowed for equilibration before centrifugation was started), calculated in the form of a Scatchard plot, is given in Fig. 2. The curve is strongly concave upwards, and as a first approximation, the data can be interpreted in terms of specific and non-specific binding, the latter being considered as a partitioning of the aurovertin between the medium and the membrane phase (see ref. 13 for a more detailed description of non-specific binding). Graphically this binding is expressed as a linear curve, parallel to the abscissa, with a constant value of bound/free. Since in this case it is not possible to eliminate the specific binding without influencing the system (as could be done in the system described in ref. 13), a best-fit solution was obtained by assuming various values of bound/free for the non-specific binding and subtracting this graphically (see refs. 13 and 14) from the experimetnal curve. When a value of 0.2 was assumed for bound/free for this non-specific boinding, a linear Scatchard plot was obtained for the specific binding, cutting the abscissa at a point corresponding to a concentration of specific binding sites of 0.81 nmol/mg protein (see Fig. 2). This is twice the value derived from Fig. 1. Although it was possible to simulate the experimentally obtained binding curve with values of bound/free less then 0.2 (the resulting corrected

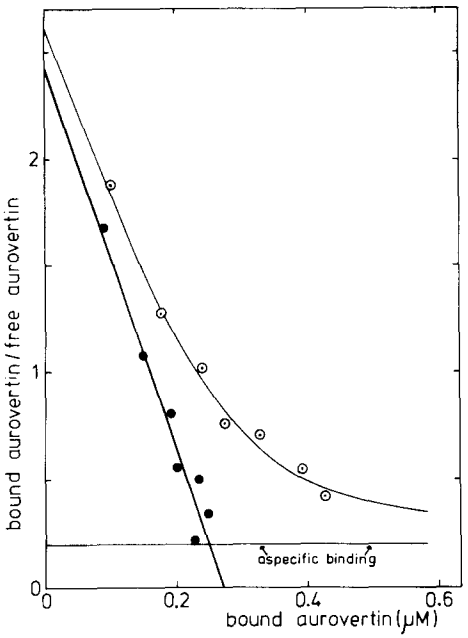

Fig. 2. Binding of aurovertin to A particles. A particles (0.333 mg/ml), suspended in 0.25 M sucrose/10 mM Tris-HCl buffer (pH 8.0), were incubated for 30 min with varying amounts of aurovertin. After the incubation the particles were spun down at 150 000 × *g* and the aurovertin in the supernatant was measured fluorimetrically in the presence of excess F_1. The binding curve (○———○) was corrected for the contribution of the 'non-specific' binding as described in the text. The resulting binding curve for the specific binding (●———●) gives the following binding parameters: K_D = 0.11 μM; n = 0.81 nmol/mg protein.

Scatchard is then not linear) the concentration of specific binding sites in that case exceeded twice the F_1 concentration. It was not possible to simulate the binding curves with any value of bound/free for non-specific binding, on the assumption that the concentration of specific sites equals the F_1 concentration.

Completely analogous results were obtained for T particles and sucrose-particles and also for the ATPase complex. The latter was first dialyzed to remove all residual detergent so that the complex became insoluble. The results of an experiment with the ATPase complex are shown in Fig. 3. Also here non-specific binding apparently occurs and correction for this type of binding results in a linear Scatchard plot for the residual (specific) binding. The K_D for the specific binding (0.1 μM) is the same as that obtained with particles, and the concentration of binding sites (2.9 nmol/mg) is also twice the value found by the fluorimetric method.

Binding of aurovertin to LiCl-treated submitochondrial particles and ATPase complex. An alternative approach was to extract the β-subunits from the F_1 on the particles by LiCl treatment and to titrate the extract with aurovertin. Verschoor et al. [9] have shown, using this method with the isolated F_1, that the aurovertin-binding site is on the β-subunit and that F_1 contains two β-subunits. After treatment of a particle suspension with 0.85 M LiCl for 3 h, causing complete loss of ATPase activity, the particles were spun down, and both

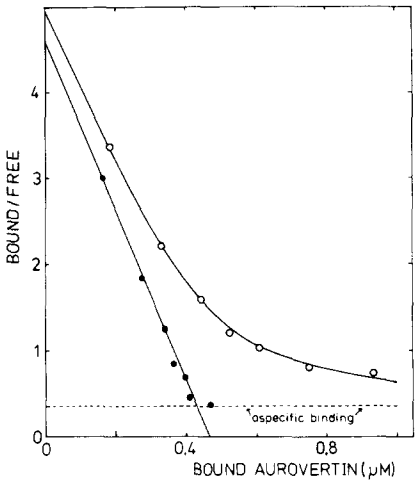

Fig. 3. Binding of aurovertin to the ATPase complex. The ATPase complex was dialyzed to remove residual detergent, and the binding of aurovertin was measured as described in Fig. 2. The incubation mixture contained 0.163 mg protein/ml. After correction of the binding curve (○———○) for the 'non-specific' binding, a linear Scatchard plot for the specific binding was obtained (●———●). The parameters are: K_D = 0.103 μM; n = 2.88 nmol/mg protein.

the precipitate and the supernatant were tested for aurovertin binding sites.

Since the resuspended precipitate did not show an appreciable enhancement of the fluorescence of added aurovertin, it was concluded that all, or nearly all,

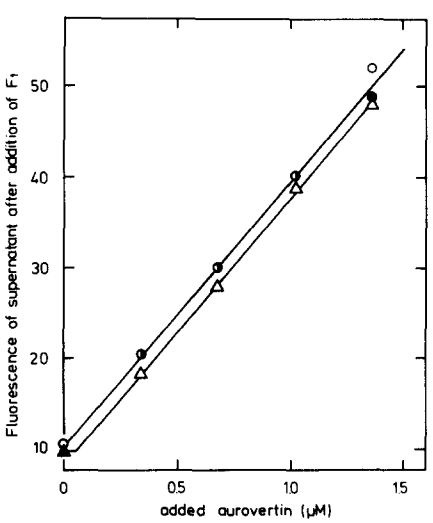

Fig. 4. Binding of aurovertin to particles extracted with LiCl. T particles (6 mg/ml) were treated with 0.85 M LiCl for 3 h at 20°C and then centrifuged. The pellet was suspended in 0.25 M sucrose/10 mM Tris (pH 8) and after 6-fold dilution relative to the original suspension treated with various amounts of aurovertin. Control tubes contained the same amounts of aurovertin but no protein. After centrifugation the aurovertin content of the supernatants was measured in the presence of excess F_1. Since the curve for the control (○———○) coincides with the calibration curve (●———●), no aurovertin is lost by binding to the centrifuge tubes. The curve obtained in the presence of particles (△———△) shows a small specific binding (0.045 μM) but no non-specific binding.

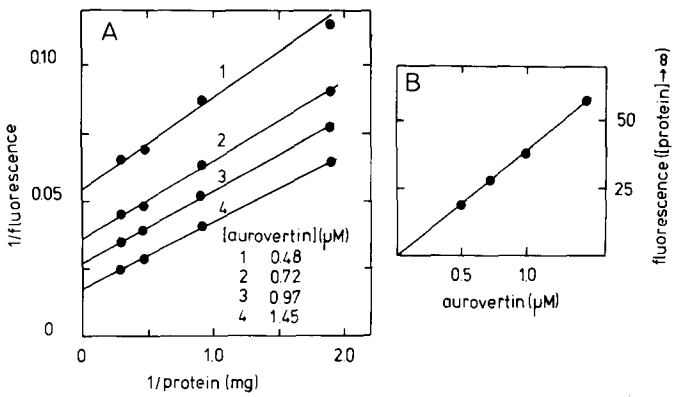

Fig. 5. Binding of aurovertin to LiCl-treated submitochondrial particles. Submitochondrial particles were treated for 3 h with 0.85 M LiCl as described in ref. 9, in the absence of ATP. After centrifugation, samples of the supernatant were titrated with aurovertin. The protein concentrations given refer to the original protein content (before centrifugation). The points in B are the inverse of the intersections of the curves in A with the ordinate. The fluorescence of bound aurovertin is 35 times the fluorescence of free aurovertin.

aurovertin binding sites had been extracted. To quantitate this result more precisely, we performed the binding experiment shown in Fig. 4. The concentration of 'specific' binding sites (no non-specific sites were revealed in this experiment) equals 0.045 nmol/mg. i.e. 4% of the original concentration (1.1 nmol/mg). This 4% of residual sites can largely be accounted for by the amount of solvent retained in the pellet after centrifugation of the LiCl-treated particles. The direct conclusion must be that the extraction of aurovertin-binding sites with LiCl is complete. Fluorimetric titrations were carried out with the supernatant fraction. The fluorescence enhancement of aurovertin on binding to this supernatant (Fig. 5) was identical with that found for binding to the isolated β-subunit [9], viz. 30—35-fold. The dissociation constants, derived from the

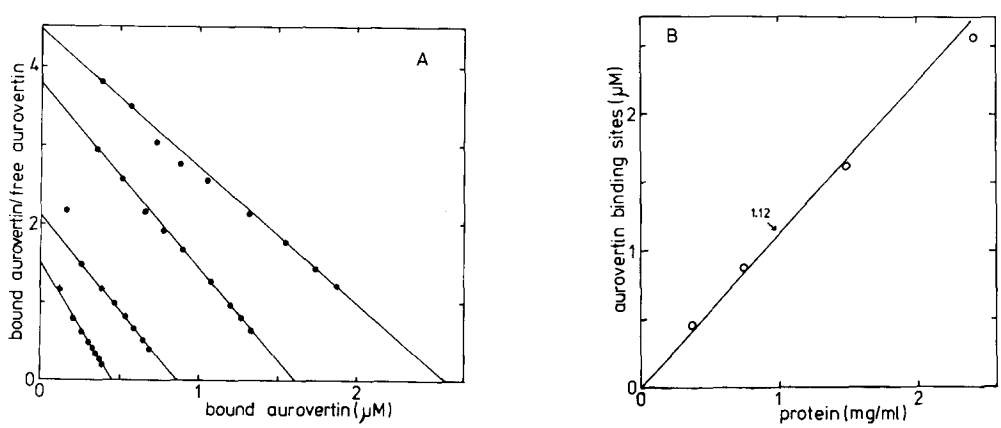

Fig. 6. Binding of aurovertin to LiCl-treated submitochondrial particles. The fluorescence of bound aurovertin (Fig. 5) and free aurovertin were used to calculate the concentrations of bound and free aurovertin for all points shown in Fig. 5A. From the resulting Scatchard plots K_D values between 0.3 and 0.6 μM can be derived. The concentration of binding sites is linearly dependent on the protein concentration as shown in B: n = 1.12 nmol/mg protein.

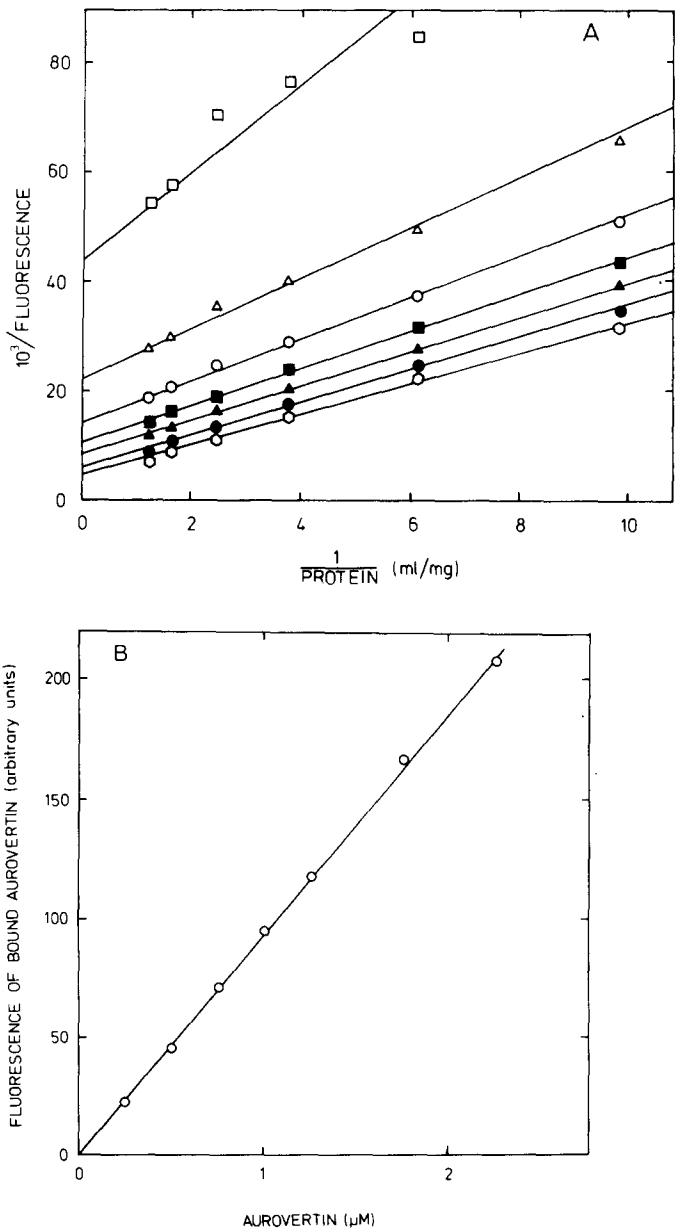

Fig. 7. Binding of aurovertin to LiCl-treated ATPase complex. The experiment was carried out essentially in the same way as described in Fig. 5. After incubation with LiCl, however, the insoluble material was not removed since it did not influence the fluorescence measurements. The fluorescence enhancement upon binding of aurovertin was 32-fold.

Scatchard plots in Fig. 6 vary somewhat with the protein concentration, lying between 0.3 and 0.6 μM. These values are similar to those of the aurovertin complexes with dissociated F_1 or isolated β-subunit [9]. The concentration of binding sites (= β-subunits) is 1.12 nmol/mg of the original particles, corresponding to 0.56 nmol F_1/mg protein.

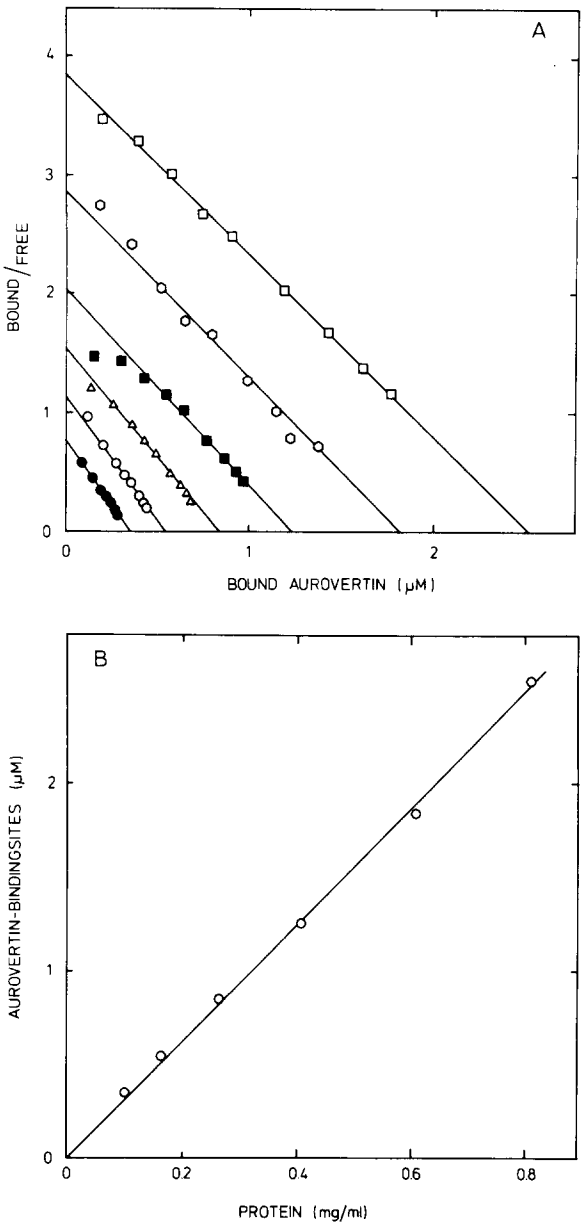

Fig. 8. From the values for the fluorescence of bound and free aurovertin the concentration of bound and free aurovertin for each point of the titrations of Fig. 7 were calculated. The resulting Scatchard plots (A) show a K_D value of approximately 0.6 μM. The concentration of binding sites is proportional to the protein concentration (B): n = 3.05 nmol/mg protein.

The data obtained with the ATPase complex are presented in Figs. 7 and 8. In the experiment shown the LiCl-treated complex was tested directly, without removal of insoluble material by centrifugation. Using the supernatant fraction after centrifugation gave identical results. The fluorescence enhancement is

again 35-fold, the K_D about 0.6 μM and the concentration of sites 3.05 nmol/mg, corresponding to 1.52 nmol F_1/mg protein of the original complex.

Discussion

In Table I, the concentrations of F_1, aurovertin-binding sites (determined both by fluorimetric titration and direct binding) and β-subunits in submitochondrial particles and the ATPase complex are compared. The direct fluorimetric titrations of both submitochondrial particles and the ATPase complex yield a concentration of aurovertin-binding sites equal to only half that measured by fluorimetric titrations of the extracted β-subunits or by direct binding experiments. The reason for this is not clear.

The possible sources of error in a direct fluorimetric titration are insufficient to explain this difference. It is tempting to consider that the factor one-half is not coincidental. At first sight it might appear that a simple explanation is that only one of the two aurovertin-binding sites in F_1 bound in the complex of the particles has a conformation conducive to fluorescence enhancement on aurovertin binding. However, this is difficult to reconcile with the indications of independent and identical sites by the direct binding experiments. Although models can be proposed to explain the peculiar fluorimetric titration of particles and ATPase complex with aurovertin, they are too speculative in the absence of any evidence in favour of their physical meaningfullness.

Paradoxically, the conclusion that the concentration of F_1 in mitochondria is the same as that of QH_2 : cytochrome *c* oxidoreductase, determined by antimycin titration, was made on the assumption that F_1 contains one aurovertin-binding site [5]. Although it is now clear that it contains two binding sites, the conclusion was correct since aurovertin titrates only one site under the conditions of this previous work. The T particles that contained 0.56 nmol F_1/mg protein according to the experiments described, contained also 0.56 nmol inhibitory antimycin-binding sites (not shown).

The ATPase complex isolated by the method described previously [4] contains 1.52 nmol F_1/mg, corresponding to a minimum molecular weight (protein

TABLE I

F_1 CONTENT AND AUROVERTIN BINDING IN SUBMITOCHONDRIAL PARTICLES AND ATPase COMPLEX

(a), fluorimetric titration; (b), 'specific' binding sites.

Preparation	Concentration (nmol/mg protein) of			
	F_1 *	aurovertin-binding sites		β subunits
		(a)	(b)	
A particles	0.35	0.4	0.81	—
T particles	0.56	0.56	1.1	1.12
Sucrose particles	—	0.54	—	1.1
ATPase complex	—	1.5	2.9	3.05

* Ref. 5.

basis) of the complex of 660 000, but the preparation still contains impurities. The purer oligomycin-insensitive complex [4] has a minimum molecular weight of 450 000.

Of the three methods described to determine the concentration of aurovertin-specific binding sites, and therefore of F_1, the fluorimetric titration of the LiCl-solubilized β-subunits is the most convenient one for routine determinations. It is a rapid, simple and reliable method. The direct fluorimetric titrations have the disadvantage that each titration takes a very long time (more than 15 min per addition) and the interpretation is not clear. The direct binding experiments are not very accurate since the correction for non-specific binding introduces an uncertainty and large amounts of F_1 are needed for the measurement of free aurovertin.

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