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THE BINDING OF AUROVERTIN TO ISOLATED F_1 (MITOCHONDRIAL ATPase)

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SUMMARY

1. Isolated F_1 contains 14.9 % N, indicating the presence of at least 8 % non-protein material. The Lowry method, standardized with bovine serum albumin, correctly measures the protein content.

2. An extinction coefficient of 28.5 $mM^{-1} \cdot cm^{-1}$ at 367.5 nm was found for aurovertin D in ethanol.

3. The fluorescence enhancement of aurovertin bound to F_1 at pH 7.5 was found to be more than 100-fold.

4. Binding parameters calculated from the fluorescence enhancement with fixed F_1 and variable aurovertin concentrations, and vice versa, indicate two binding sites per F_1 molecule.

5. The fluorescence data are not readily interpreted on the basis of successive binding of aurovertin by 3-component binding reactions of the form $E+A \rightleftharpoons EA$, but fit closely a model of two non-interacting sites binding aurovertin in a 4-component reaction, $EF+A \rightleftharpoons EA+F$, with an equilibrium constant of about 2.

INTRODUCTION

It is clearly established that the ATPase (Coupling Factor 1 or F_1), first extracted by Racker and co-workers from beef-heart mitochondria [1], is the terminal enzyme of oxidative phosphorylation, catalysing the reaction

$$ADP+P_i+\text{energy} \rightleftharpoons ATP+H_2O \quad (1)$$

the energy being derived from electron-transfer reactions that also take place in the same membrane. Analogous enzymes, with somewhat similar subunit structure, have also been isolated from chloroplast (reviewed in ref. 2) and bacterial membranes (reviewed in ref. 3).

The exact nature of the link between the electron-transfer reactions and the

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ATP-synthesizing reaction is still under active investigation. According to the chemiosmotic hypothesis [4], the function of the electron-transfer reactions is to provide protons at a high electrochemical activity on the outside of the mitochondrial membrane (inside of the chloroplast thylakoid membrane), and these protons are fed back through a proton channel to the F_1 which is located on the inside of the mitochondrial membrane (outside of the thylakoid membrane) in such a way that the reaction illustrated in Eqn. 1 is driven to the right. Strong support for this hypothesis has been given by the demonstration, in several different types of experiments, that a suitable proton gradient can drive the synthesis of ATP, at a rate consistent with this process being on the main pathway of the oxidative phosphorylation reaction (reviewed in ref. 5). It cannot be excluded, however, that a more direct link between the electron-transferring and ATP-synthesizing systems exists in addition to that provided by the translocation of protons across the membrane. This could be, for example, a proton channel within the membrane [6], or a direct interaction between polypeptide chains in the electron-transfer system with those in the ATP-synthesizing system [7, 8]. If this were the case, one would expect to find a stoicheiometric relationship between the systems [cf. 9]. If, as envisaged in the chemiosmotic hypothesis, the only link is via protons outside the membrane, no stoicheiometric relationship would a priori be expected.

Bertina et al. [10] have reported that in both rat-heart and rat-liver mitochondria the concentration of F_1, measured by aurovertin binding, is equal to the concentration of the bc_1 complex, measured by antimycin binding. This conclusion was based on the assumption that F_1 contains only one aurovertin-binding site under the conditions used. Later work has, however, cast doubt on the validity of this assumption. Scatchard plots seemed clearly to show the presence of two types of binding sites in submitochondrial particles [11]. Chang and Penefsky [12] reported that beef-heart F_1 combines with 2 molecules of aurovertin with equal binding constants in the presence of ATP, but only one in its absence, and Lardy et al. [13] have reported that rat-liver mitochondrial ATPase binds 2 molecules of aurovertin per molecule (360 000 daltons), and the beef-heart enzyme an average of 1.25 molecules of aurovertin. The lower binding of the beef-heart enzyme was ascribed to dissociation of this preparation into its sub-units.

In view of the importance of establishing the stoicheiometry between F_1 and the electron-transfer chain, we have reinvestigated the binding of aurovertin to purified beef-heart F_1. Particular attention was given to the question of determining the concentrations of F_1 and aurovertin. We now find that isolated beef-heart F_1 binds two molecules of aurovertin, even in the absence of ATP. We present evidence that the binding sites are non-interacting and that binding of aurovertin leads to dissociation of a component of F_1, according to the equation $EF+A \rightleftharpoons EA+F$.

METHODS

F_1 was prepared according to the method of Knowles and Penefsky [14] and taken up in 250 mM sucrose, containing 10 mM Tris, 2 mM EDTA and 4 mM ATP brought to pH 7.5 with acetic acid. Samples were stored at the temperature of liquid nitrogen. After thawing a sample, the protein was precipitated with an equal volume of neutralized saturated ammonium sulphate, collected by centrifugation and dis-

solved in 250 mM sucrose, 10 mM Tris and 2 mM EDTA brought to pH 7.5 with acetic acid. The precipitation with ammonium sulphate was repeated 4 times. The samples of F_1 used contained 2.0 mol ATP and 0.9–1.1 mol ADP per mol F_1 [cf. 15, 16]. According to acrylamide-gel electrophoresis both in the presence and absence of dodecyl sulphate, the F_1 was more than 90 % pure. The concentration of F_1 is given in μM, based on the protein concentration (see below), assuming 100 % purity and that a molecule of F_1 contains 319 kilodalton protein (see Discussion).

Aurovertin used in the experiments reported was a sample of aurovertin D isolated by Bertina [17]. A sample of aurovertin kindly supplied by Dr. H. S. Penefsky gave essentially the same results. Aurovertin solutions were freshly prepared in ethanol, the concentration of which was determined spectrophotometrically, using $A_{367.5\ nm} = 28.5\ mM^{-1} \cdot cm^{-1}$ (see below). The ethanol concentration in the aurovertin titrations did not exceed 3%.

Fluorescence measurements were made at 22 °C with a Perkin-Elmer MPF-2 fluorimeter equipped with a stirring compartment, with 368 nm as excitation wavelength and 480 nm as emission wavelength. The band widths were 4 nm.

F_1-bound ATP and ADP were determined as described by Harris et al. [15].

RESULTS

Determination of the concentration of F_1.

In the literature, the concentration of F_1 has been usually expressed as dry weight of the dialysed enzyme, either measured directly [18] or indirectly from the protein concentration determined by refractometry [12, 18, 19], spectrophotometry [14, 18, 19], turbidity [14], or by the biuret [20] or Lowry [19] or modified Lowry [20, 21] methods, and standardized with dry enzyme. In earlier papers from this laboratory [15, 22], we have used the Lowry method but, as pointed out elsewhere [16], used an incorrect $A_{280\ nm}{}^{1\%}$ to standardize the serum albumin.

Four methods of determining the concentration of F_1 were compared, viz. dry weight, Kjeldahl N determination, biuret and Lowry. For determination of the dry weight, 2 ml of F_1 dissolved in 250 mM [^{14}C]sucrose (10 μCi), containing 10 mM Tris, 2 mM EDTA and 4 mM ATP, brought to pH 7.5 with acetic acid, was dialysed against 1 l water, the dialysis fluid being changed twice within 24 h. No radioactivity ($< 3 \cdot 10^{-5}$ μCi) could be detected in the last dialysate. The F_1, which was precipitated by this procedure, was washed out of the dialysis bag, the washings freeze-dried and finally dried over P_2O_5 to constant weight. The N content was determined in two laboratories by the Dumas procedures, one gasometric, the other gas chromatographic. Both procedures gave the identical result, namely 14.9 % N, i.e. 92 % (14.9 · 100/16.2) of that expected from the N content calculated from the amino acid composition [23]. The biuret [24] and Lowry [25] methods, standardized with bovine serum albumin ($A_{279\ nm} = 6.67\ cm^{-1}$ for a 1 % solution [26]), yielded 91 and 95 %, respectively, of the dry weight.

The protein content of a sample of F_1 dialysed, after thawing, against 20 mM potassium phosphate buffer (pH 7.6), 50 mM KCl and 0.5 mM EDTA, was determined by the Kjeldahl (protein = 100 N/16.2), the biuret and the Lowry methods. The values obtained by the biuret and Lowry procedures were 102.5 and 99.5 %, respectively, of that obtained by the Kjeldahl.

It is concluded that both the biuret and the Lowry methods correctly determine the protein content within 3 %. Since the latter requires less material, it was used routinely.

Determination of the concentration of aurovertin

In previous papers from this laboratory [10, 27, 11], the concentration of aurovertin in stock ethanolic solutions was determined using the absorbance coefficient $A_{367.5\ nm} = 42.7\ mM^{-1} \cdot cm^{-1}$ [28]. This value has also been used by Chang and Penefsky [12]. Mulheirn et al. [29] have, however, reported a considerably lower value, viz. $A_{367.5\ nm} = 34.5\ mM^{-1} \cdot cm^{-1}$, for aurovertin B, which differs from aurovertin D only in the replacement of a side-chain secondary alcoholic hydroxyl by a hydrogen atom. Bertina [17] has also reported for his purest preparation a lower value ($A_{367.5\ nm} = 29\ mM^{-1} \cdot cm^{-1}$) than that of Baldwin et al. [28], but we had ascribed this to impurities in his preparation. However, the report of Mulheirn et al. [29] prompted us to request Mr. C. Kruk and Mr. J. C. van Velzen of the Organic Chemistry Department of the University of Amsterdam to purify our preparation of aurovertin D further and to determine its absorbance coefficient. The value obtained, $A_{367.5\ nm} = 28.5\ mM^{-1} \cdot cm^{-1}$, was almost identical with that reported by Bertina, and we have used this value in this paper.

Binding of aurovertin to F_1

Fig. 1 shows the increase of fluorescence obtained by adding increasing

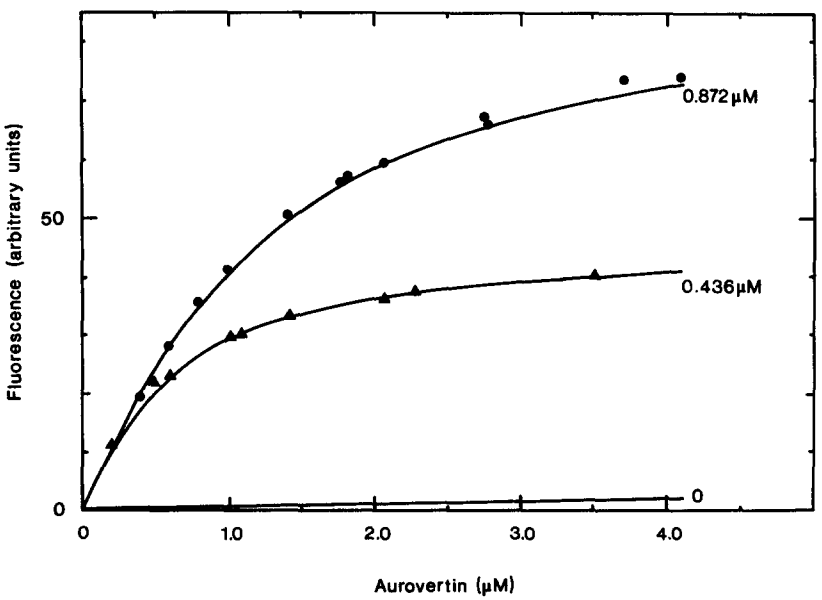

Fig. 1. Aurovertin titration curves of F_1. The observed fluorescence is plotted against the aurovertin concentration with the indicated concentrations of F_1 dissolved in 250 mM sucrose, 10 mM Tris-acetate buffer, 2 mM EDTA, pH 7.5 at 22 °C. The continuous lines represent the theoretical curves on the basis of a four-component equilibrium (see text), with $K = 2.1$; α_b and $\alpha_f = 57.5$ and 0.4 arbitrary units $\cdot\ \mu M^{-1}$, respectively; number of binding sites: $n = 1.74$.

amounts of aurovertin to two different concentrations of F_1 dissolved in a solution containing 250 mM sucrose, 10 mM Tris and 2 mM EDTA brought to pH 7.5 with acetic acid. As previously found by Chang and Penefsky [12] and Yeates [27], the increase of fluorescence is biphasic in time. The fluorescence recorded in Fig. 1 is the maximum value reached with each concentration of aurovertin. The measured fluorescence, F_{obs}, is a function of the amount of bound and unbound aurovertin, according to Eqn. 2.

$$F_{obs} = \alpha_f a_f + \alpha_b a_b \tag{2}$$

where α_f and α_b are the fluorescence coefficients (arbitrary units $\cdot$ μM^{-1}) of free and bound aurovertin, respectively, and a_f and a_b (μM) are the concentrations of free and bound aurovertin, respectively. In the range of aurovertin concentrations used, no "inner filter" quenching of the fluorescence by the aurovertin was detectable. Eqn. 3 is derived from Eqn. 2:

$$F_{obs} = (\alpha_b - \alpha_f)a_b + \alpha_f a \tag{3}$$

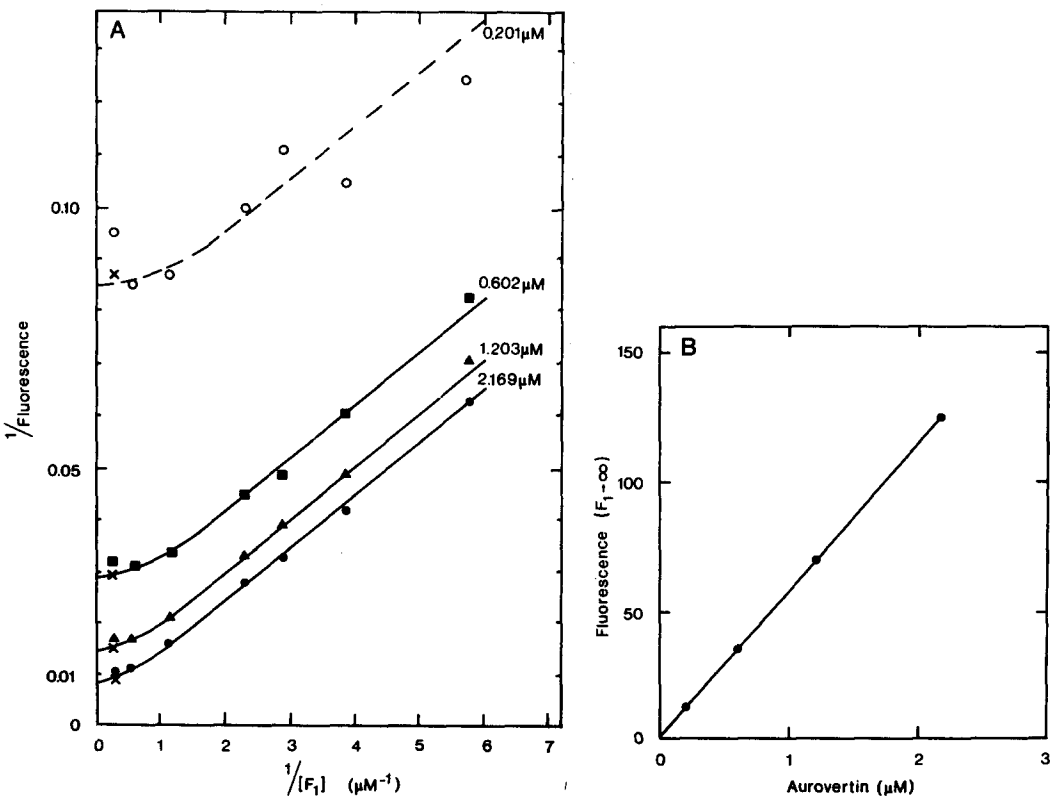

Fig. 2. (A) Double-reciprocal plot of fluorescence against F_1 concentration at the indicated concentrations of aurovertin. Reaction conditions as described in Fig. 1. The points × represent the observed fluorescence after correction for non-linearity between fluorescence and protein concentrations at higher protein concentration (see Fig. 3). The lines are drawn by hand. (B) Fluorescence at infinite protein concentration ($F_{F_1 \to \infty}$) plotted against aurovertin concentration. A value of $\alpha_b = 57.5$ units $\cdot$ μM^{-1} is obtained from this figure.

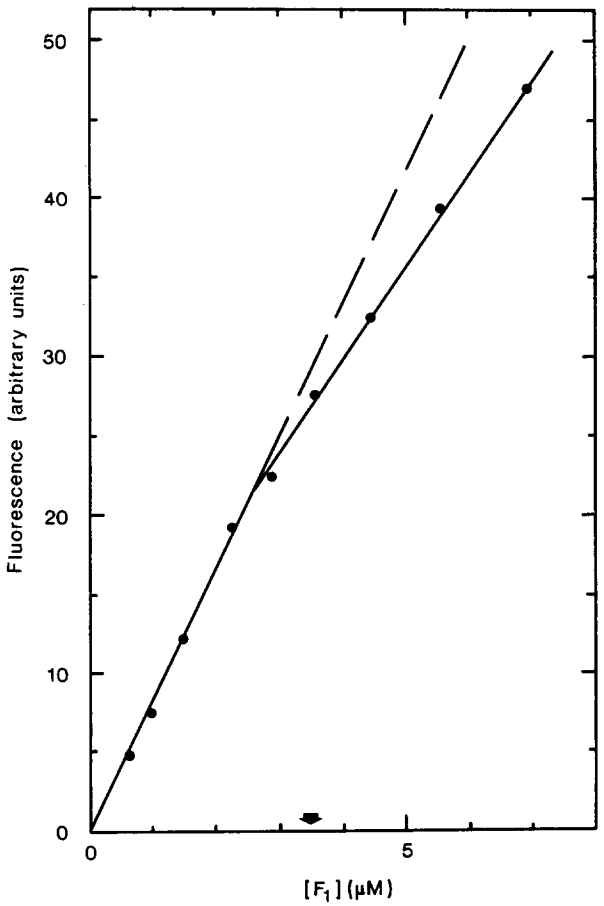

Fig. 3. Relation between fluorescence and protein concentration at constant protein/aurovertin ratio, to show the non-linearity between fluorescence and protein-aurovertin complex concentration at high protein concentrations. [Aurovertin]/[F_1] ratio, 17.3; otherwise conditions as in Figs. 1 and 2. The arrow indicates the highest protein concentration used in the experiment given in Fig. 2.

where $a = a_f + a_b$ is the total concentration of aurovertin, from which follows that

$$a_b = \frac{F_{obs} - \alpha_f a}{\alpha_b - \alpha_f} \tag{4}$$

α_f, determined by measuring the fluorescence in the absence of F_1 (see Fig. 1), was found to be 0.4 units $\cdot$ μM^{-1}, under the conditions of the experiment shown.

α_b was determined by measuring the fluorescence increase at various protein concentrations and fixed aurovertin concentration (Fig. 2A), and plotting the inverse of F_{obs} against the inverse of the protein concentration [cf. 30]. At the highest F_1 concentration (3.46 μM) it was necessary to correct for quenching of the fluorescence by the protein (see Fig. 3). The corrected points are indicated in Fig. 2A. The points lie on parallel straight lines at low F_1 concentration but deviate at high concentrations. This gives some difficulty in extrapolating to infinite F_1 concentration ($1/[F_1] = 0$),

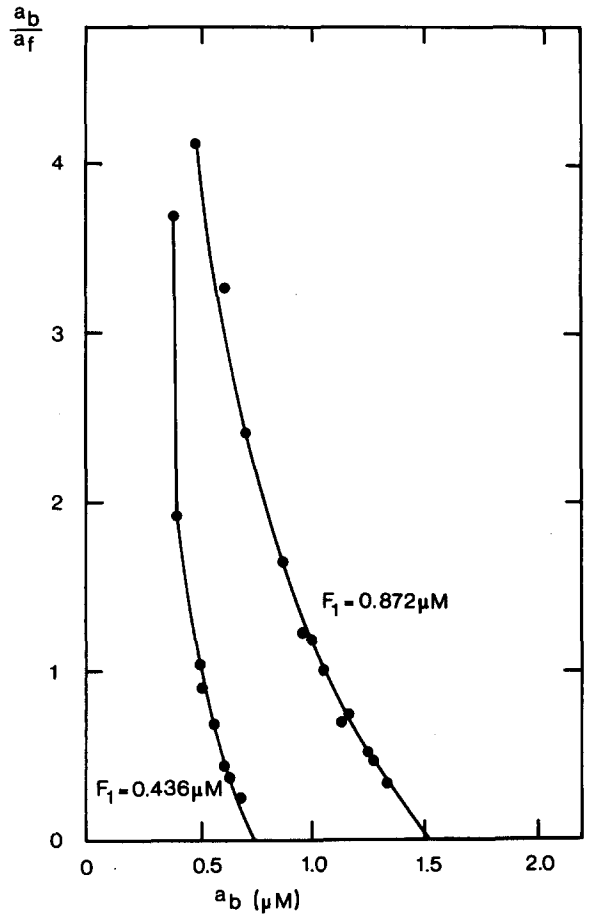

Fig. 4. Scatchard plot for the binding of aurovertin to F_1 at two different protein concentrations, calculated from the data of Fig. 1.

but the points are sufficiently close to the ordinate to allow the extrapolation to be carried out with reasonable accuracy. The intercept on the ordinate plotted against the total aurovertin concentration (Fig. 2B) yields a straight line, the slope of which is equal to α_b, since at infinite protein concentration, all the aurovertin must be bound. Under the conditions of these measurements and that of Fig. 1, $\alpha_b = 57.5$ units · μM^{-1}. Substituting the values for α_f and α_b in Eqn. 4, a_b can be calculated for each value of a, and $a_f = a - a_b$ can then be calculated, and a Scatchard plot constructed. This is given in Fig. 4.

Calculation of parameters for binding of aurovertin to F_1

The Scatchard plots shown in Fig. 4 are curved, concave upwards, with intercepts on the abscissa corresponding to two binding sites per molecule of F_1. The simplest interpretation of the Scatchard plot would seem to be to assume two binding sites per molecule of F_1 with different dissociation constants. The binding data of Fig. 1 could be simulated assuming values of K_1 and K_2 (dissociation constants) of 0.02 and

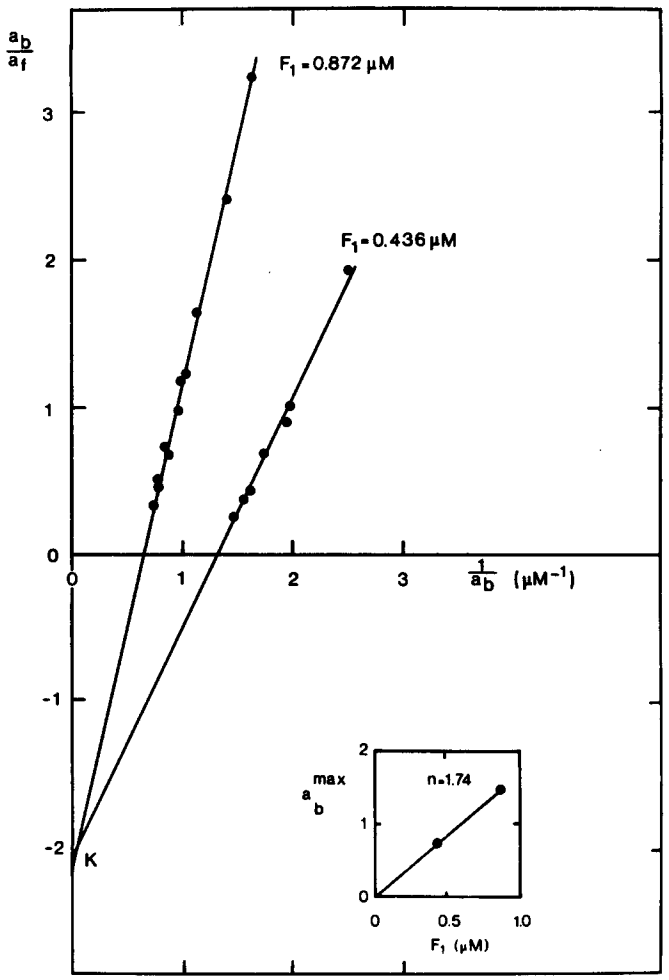

Fig. 5. Plot of bound aurovertin/free aurovertin against the inverse of bound aurovertin (see Appendix, Eqn. 8) for the binding of aurovertin to F_1. In the inset, the amount of maximally bound aurovertin is plotted against the protein concentration. Total number of binding sites (n) derived from this figure is 1.74.

0.8 μM, respectively, for the lower concentration of F_1, and 0.08 and 1.2 μM, respectively, from the data with the higher concentration. (These simulations are not those shown in Fig. 1, see below.) The simulated curve was quite sensitive to changes of K_2 (within 0.1 μM) but relatively insensitive to changes in K_1. The fact that K_1 and K_2 are dependent on the concentration of F_1 (as is immediately apparent by the fact that the two curves in Fig. 4 are not parallel) throws doubt on this simple interpretation of the curved Scatchard plot. Moreover, it was not found possible closely to simulate double-reciprocal plots shown in Fig. 2A on the basis of a simple three-component binding reaction of the form $E+A \rightleftharpoons EA$ even if there are two binding sites (see Appendix).

As shown in the Appendix, however, the data fit a simple four-component

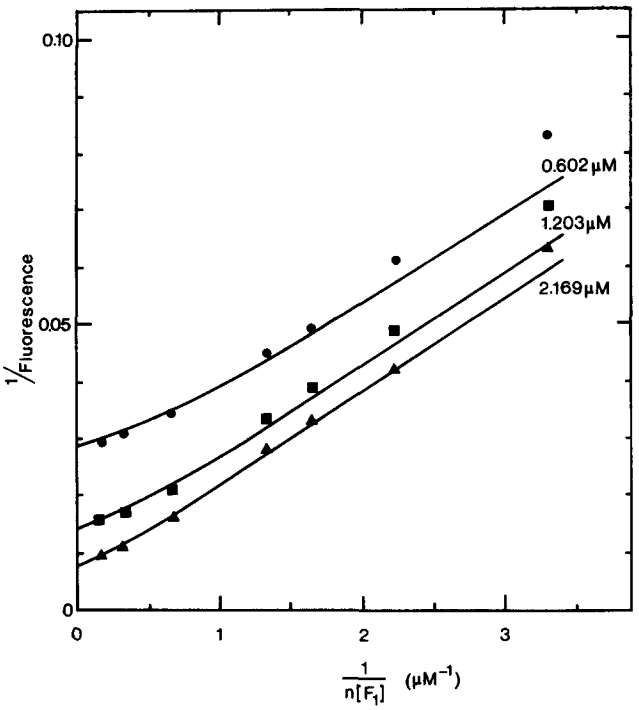

Fig. 6. Simulation of the experiment shown in Fig. 2A on the basis of a four-component equilibrium of the binding of aurovertin to F_1. The following constants were used: $K = 2.1$, $\alpha_b = 57.5$ units $\cdot \mu M^{-1}$ and $\alpha_f = 0.3$ units $\cdot \mu M^{-1}$. The aurovertin concentrations used are indicated in the figure. The experimental points are those shown in Fig. 2A, but plotted on an abscissa scale of concentration of binding sites ($= n[F_1]$), on the basis of $n = 1.74$, as derived from Fig. 5.

TABLE I

PARAMETERS OF BINDING OF AUROVERTIN TO ISOLATED F_1

The fluorescence data, plotted as in Fig. 1, have been interpreted either on the basis of three-component binding with 2 binding sites: $E+A \rightleftharpoons EA$; $K_1 = [E][A]/[EA]$; $E'+A \rightleftharpoons E'A$; $K_2 = [E'][A]/[E'A]$; or four-component binding: $(EF)_2+A \rightleftharpoons EA \cdot EF+F$; $EA \cdot EF+A \rightleftharpoons (EA)_2+F$; intrinsic $K = [EA][F]/[EF][A]$. n = [aurovertin-binding sites]/[F_1].

Preparation	Bound nucleotides (mol/mol F_1)		ATPase activity (μmol/min per mg protein)	F_1 (μM)	n	3-component model		4-component model
	ATP	ADP				K_1 (μM)	K_2 (μM)	K
1*	2.04	1.06	89	0.436	1.74	0.04	0.8	1.96
				0.872	1.74	0.08	1.2	2.08
2	—	—	97	0.515	1.74	0.09	1.4	1.43
				1.027	1.75	0.13	2.0	1.59
3	1.95	0.89	87	0.565	1.78	0.03	0.4	5

* Data given in Figs. 1–6.

binding reaction of the form $EF+A \rightleftharpoons EA+F$ (Eqn. 5) which yields a straight-line plot of bound ligand/free ligand against 1/bound ligand, intercepting the abscissa at the reciprocal of the concentration of binding sites and the ordinate at minus the equilibrium constant. Fig. 5 shows that the data of Fig. 1 obey the equation closely (the points at the beginning of the titration, where the values for free aurovertin are subject to large errors, have been omitted). The intercepts of the lines drawn in Fig. 5 intercept the abscissa at a concentration of binding sites close to double that of F_1, and the ordinate at $K = 2.08$ and 1.96, respectively. The lines in Figs. 1 and 6 are calculated according to the equilibrium given by Eqn. 5, assuming the values for K and the concentration of binding sites found from Fig. 5.

In Table I are assembled data for two other titrations calculated on the basis of both the 3-component and 4-component binding reactions.

DISCUSSION

Nitrogen content and molecular weight of F_1

The N content of F_1, 14.9 %, is 8 % less than that calculated from the amino acid composition given by Knowles and Penefsky [23], namely 16.2 % N, indicating the presence of about 8 % non-protein material in the F_1. The nature of the non-protein material has not yet been established. Nor is it known if it is an intrinsic part of the protein or a contaminant. The ATP, ADP and Mg contents [16] account for only about 0.5 % of the dry weight.

The Kjeldahl, Lowry and biuret methods all gave the same value for the protein concentration within 3 %. According to our data, the protein concentration should be multiplied by $16.2/14.9 = 1.09$ to obtain the dry weight. This is considerably different from the factor used by Garrett and Penefsky [19] to convert Lowry protein to dry weight, namely $1/1.18 = 0.85$. Thus, we differ from Garrett and Penefsky by the factor of 1.28 in converting Lowry protein to dry weight.

The molecular weight of F_1 has been determined by several laboratories by the sedimentation equilibrium method. Forrest and Edelstein [31] found 280 000 for the heavier component, Lambeth et al. [32] 310 000 as the weight average and Knowles and Penefsky [23] found 347 000. Lambeth et al. [32] found 360 000 by gel filtration. If the 8 % of non-protein material that we find in F_1 was present in the preparations used for the determination of the molecular weight [31, 32, 23], these values should be decreased by 8 % to obtain the number of daltons of protein in one molecule, e.g. Knowles and Penefsky's molecular weight corresponds to a 319 kilodalton protein. This is close to the value of 323 kilodaltons calculated on the basis of a sub-unit composition of F_1, $\alpha_2\beta_2\gamma_2\delta_2\varepsilon_2$ and the molecular weights of the subunits determined by sedimentation equilibrium or amino acid analysis [23].

Binding of aurovertin to isolated F_1

On the basis of a protein molecular weight of 319 000 and an absorbance coefficient for aurovertin at 367.5 nm of 28.5 $mM^{-1} \cdot cm^{-1}$, our data indicate close to 2 binding sites per F_1 molecule. However, the calculation of the number of binding sites is dependent upon the correct values for the molecular weight of F_1, the absorbance coefficient of aurovertin and the purity of the preparation. If the protein molecular weight is higher than 319 000, the number of binding sites will be correspondingly

higher. If an $A_{367.5\ nm} = 34.5\ mM^{-1} \cdot cm^{-1}$ [29] were used instead of the $A_{367.5\ nm}$ of 28.5, the number of binding sites would be 17 % less. The presence of contaminating proteins in the F_1 preparation (probably less than 10 %) would increase the number of binding sites. If Lardy et al. [13] used an $A_{367.5\ nm} = 42.7\ mM^{-1} \cdot cm^{-1}$, the number of binding sites of 1.25 that they obtained, and ascribed to dissociation into subunits, would become 1.87 if the same value for the absorbance coefficient for aurovertin were used as in this paper. However, it then remains difficult to explain the two binding sites found with the liver enzyme, using the higher absorbance coefficient. Chang and Penefsky [12] also used the higher value, but this is largely compensated by the 28 % difference between the relation between dry weight and protein, referred to above. There is a residual 17 % difference between us and Chang and Penefsky [12] in the factor used to convert fluorescence and protein data to number of binding sites (ours higher). This is not sufficient to explain the discrepancy that Chang and Penefsky find only one binding site in the absence of ATP, whereas we find two.

Our data fit a four-component binding reaction, in which a component of F_1 (a sub-unit or a non-protein component) is dissociated simultaneously with binding of aurovertin, better than to a conventional three-component binding reaction. There is, however, no direct evidence for the aurovertin-induced dissociation of F from EF. In this sense, our interpretation of the binding data remains speculative. Both models yield two binding sites per F_1.

The isolated β sub-unit of F_1 binds aurovertin in a conventional three-component binding reaction with one binding site per molecule [33]. The increase of fluorescence on adding aurovertin to the β sub-unit is monophasic in time, compared with the biphasic kinetics with F_1 [12, 27]. On the basis of the four-component model, the biphasic kinetics with intact F_1 are readily understandable by a sequence $EF+A \rightleftharpoons EF \cdot A \rightleftharpoons EA+F$, where EA has a higher fluorescence yield than EFA. On the basis of the three-component model it could be rationalized by assuming a conformation change after binding of aurovertin, but then the rapid monophasic kinetics with the isolated β sub-unit [33] would be rather unexpected. Yeates' [27] finding that the $t_{\frac{1}{2}}$ of the slow fluorescence increase is independent of aurovertin concentration excludes the possibility that the slow phase is due to a slower combination of aurovertin to a low-affinity site.

The conclusion that isolated F_1 binds two molecules instead of one of aurovertin and the new absorbance coefficient of aurovertin requires an assessment of the determinations of the concentration of F_1 in mitochondria [10] and submitochondrial particles [11] reported earlier in this laboratory on the basis of aurovertin-binding data. These two factors alone introduce a correction factor of $42.7/(28.5 \times 2) = 0.75$. However, it was also not realized in this earlier work that the extrapolation to infinite protein concentration of the double-reciprocal plot of fluorescence against protein concentration is only valid at high protein concentrations, unless $K = 1$. If the straight portions of the lines in Fig. 2, at lower protein concentrations (cf. ref. 11), are extrapolated to infinite protein concentration the quantum yield is greatly over-estimated and the concentration of bound aurovertin (and therefore of F_1) correspondingly underestimated. Since it is difficult accurately to measure the fluorescence in the presence of high concentrations of particles, the measurement of aurovertin fluorescence is unsuitable for measuring the concentration of F_1 in particulate preparations. The measurement of the concentration of F_1-binding sites in particles stripped of F_1 [34] and

the determination of 4-chloro-7-nitrobenzofurazan-binding sites [35] are more reliable methods.

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APPENDIX

For a simple three-component binding of aurovertin (A) to enzyme (E), $E+A \rightleftharpoons EA$, the dissociation constant:

$$K = \frac{[E][A]}{[EA]} = \frac{(e-[EA])(a-[EA])}{[EA]} = \frac{(e-a_b)(a-a_b)}{a_b} \tag{6}$$

where $e = [E]+[EA]$.

When $a \gg e$, little aurovertin is bound, so that $a-a_b \cong a$, and Eqn. 6 simplifies to $a_b = ea/(K+a)$. Neglecting, as a first approximation, the fluorescence of free aurovertin ($\alpha_b/\alpha_f = 128$),

$$F_{obs} = \frac{\alpha_b e a}{K+a}$$

and

$$\frac{1}{F_{obs}} = \frac{K}{\alpha_b e a} + \frac{1}{\alpha_b e}$$

A plot of $1/F_{obs}$ against $1/e$ at low protein concentration and fixed a is a straight line passing through the origin with slope $(K/\alpha_b a)+1/\alpha_b$.

When $a \ll e$, there is little bound enzyme so that $e-a_b \cong e$ and Eqn. 6 simplifies to

$$a_b = \frac{ea}{K+e} \quad \text{and}$$

$$\frac{1}{F_{obs}} = \frac{K}{\alpha_b e a} + \frac{1}{\alpha_b a}$$

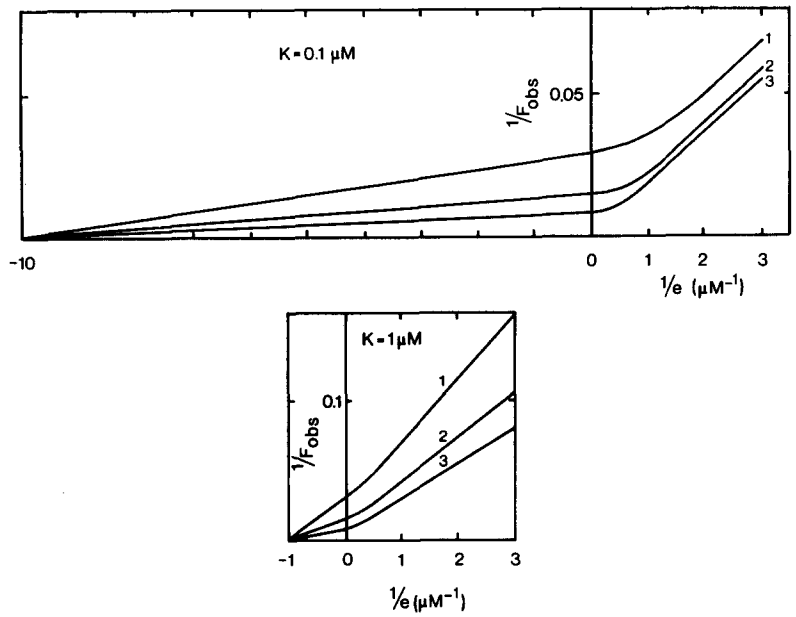

Fig. 7. Double-reciprocal plot of fluorescence (F_{obs}) against enzyme concentrations, at different fixed aurovertin concentrations, calculated for a three-component binding reaction, E+A $\rightleftharpoons$ EA. $F_{obs} = 57.5\ a_b$ where a_b is concentration of bound aurovertin (μM). e, total enzyme concentration (μM). Top: K (dissociation constant) = 0.1 μM; below: $K = 1.0\ \mu$M. Curves 1, 2, 3: total aurovertin concentration 0.6, 1.2 and 2.17 μM, respectively.

A plot of $1/F_{obs}$ against $1/e$ at high protein concentration and fixed a is a straight line, with slope equal to $K/\alpha_b e$ and intercepts on the ordinate and abscissa equal to $1/\alpha_b a$ and $-1/K$, respectively. In Fig. 7, calculated plots are shown covering the range of aurovertin and F_1 concentrations used in these experiments, assuming $K = 0.1$ and 1, respectively.

For a four-component binding of aurovertin to enzyme (EF),

EF+A $\rightleftharpoons$ EA+F, the equilibrium constant

$$K = \frac{[EA][F]}{[EF][A]} = \frac{[EA]^2}{[EF][A]} = \frac{a_b^2}{(e-a_b)(a-a_b)}, \text{ since } [F] = [EA] \qquad (7)$$

For the special case that $K = 1$,

$$a_b = \frac{ea}{e+a}, \quad F_{obs} = \frac{\alpha_b ea}{e+a}, \text{ and } \quad \frac{1}{F_{obs}} = \frac{1}{\alpha_b a} + \frac{1}{\alpha_b e}$$

A plot of $1/F_{obs}$ against $1/e$ is a straight line with slope $= 1/\alpha_b$ and intercept on the ordinate equal to $1/\alpha_b a$. Thus plots of $1/F_{obs}$ against $1/e$ at different concentrations of a will be straight lines parallel to one another. These lines are not very sensitive to changes of K between 0.2 and 10 as the calculated curves in Fig. 8 show.

Eqn. 7 may be written $K = a_b^2/(e-a_b)a_f$
from which it follows that:

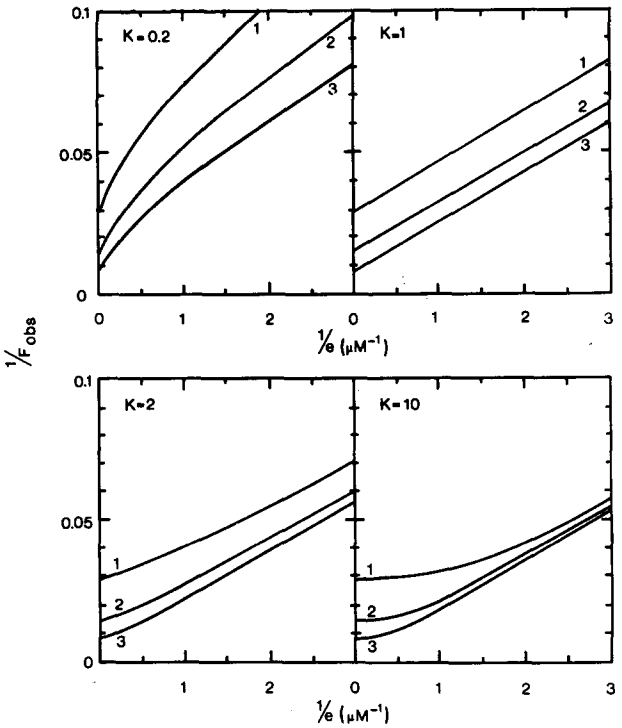

Fig. 8. Double-reciprocal plot of fluorescence (F_{obs}) against enzyme concentration at different fixed aurovertin concentrations calculated for a four-component binding reaction, EF+A $\rightleftharpoons$ EA+F. $F_{obs} = 57.5\ a_b$, where a_b is concentration of bound aurovertin (μM). e, total enzyme concentration (μM). Equilibrium constant K, as indicated in the figure. Curves 1, 2, 3: total aurovertin concentration 0.6, 1.2 and 2.17 μM, respectively.

$$\frac{a_b}{a_f} = K\left(\frac{e}{a_b} - 1\right) \tag{8}$$

which does not give a linear relation in the Scatchard plot (a_b/a_f against a_b), but a curve cutting the abscissa at e. A linear relation is, however, obtained in the plot of a_b/a_f against $1/a_b$ with an intercept on the abscissa equal to $1/e$ and on the ordinate of $-K$ (slope $= Ke$).

Indeed Fig. 4 shows non-linear Scatchard plots cutting the abscissa at a concentration of binding sites equal to double the enzyme concentrations. In accordance with Eqn. 8, the plot of a_b/a_f against $1/a_b$ is linear, the line cutting the abscissa at a value also corresponding to a concentration of binding sites equal to double the F_1 concentration (Fig. 5). This shows that F_1 contains two non-interacting identical binding sites behaving according to Eqn. 5, i.e.

$(EF)_2 + A \rightleftharpoons EA \cdot EF + F$

$EA \cdot EF + A \rightleftharpoons (EA)_2 + F$

(If K is now the intrinsic equilibrium constant of the aurovertin-binding reaction

$$[EA \cdot EF] = \frac{2Ka_f[(EF)_2]}{[F]} = \frac{2Ka_f}{a_b}[(EF)_2], \text{ since } [F] = a_b$$

$$[(EA)_2] = \frac{Ka_f[EA \cdot EF]}{2[F]} = \left(\frac{Ka_f}{a_b}\right)^2[(EF)_2]$$

$$a_b = [EA \cdot EF] + 2[(EA)_2]$$

$$= 2\frac{Ka_f}{a_b}\left(1 + \frac{Ka_f}{a_b}\right)[(EF)_2]$$

$$e = 2([(EF)_2] + (EA \cdot EF] + [(EA)_2])$$

$$= 2[(EF)_2]\left(1 + \frac{2Ka_f}{a_b} + \left(\frac{Ka_f}{a_b}\right)^2\right)$$

$$= 2[(EF)_2]\left(1 + \frac{Ka_f}{a_b}\right)^2$$

$$[(EF)_2] = \frac{e}{2\left(1 + \frac{Ka_f}{a_b}\right)^2}$$

and

$$a_b = \frac{eKa_f}{a_b\left(1 + \frac{Ka_f}{a_b}\right)} = \frac{eKa_f}{a_b + Ka_f},$$

from which it follows that $a_b/a_f = K(e/a_b - 1)$, which is the same as Eqn. 8.)

REFERENCES

1 Pullman, M. E., Penefsky, H. S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
2 Nelson, N. (1976) Biochim. Biophys. Acta 456, 314–338
3 Baltscheffsky, H. and Baltscheffsky, M. (1974) Ann. Rev. Biochem. 43, 871–897
4 Mitchell, P. (1961) Nature 191, 144–148
5 Boyer, P. D., Chance, B., Ernster, L., Mitchell, P. D., Racker, E. and Slater, E. C. (1977) Ann. Rev. Biochem., 46
6 Williams, R. J. P. (1961) J. Theoret. Biol. 1, 1–17
7 Boyer, P. D. (1965) in Oxidases and Related Redox Systems (King, T. E., Mason, H. S., Morrison, M., eds.), pp. 994–1008, Wiley, New York
8 Slater, E. C. (1974) in Dynamics of Energy-Transducing Membranes (Ernster, L., Estabrook, R. W. and Slater, E. C., eds.), pp. 1–20, Elsevier, Amsterdam
9 Ernster, L. (1975) in Enzymes: Electron Transport Systems (Desnuelle, P. and Michelson, A. M., eds.), pp. 253–276, North-Holland, Amsterdam
10 Bertina, R. M., Schrier, P. I. and Slater, E. C. (1973) Biochim. Biophys. Acta 305, 503–518
11 Van de Stadt, R. J., van Dam, K. and Slater, E. C. (1974) Biochim. Biophys. Acta 347, 224–239
12 Chang, T.-M. and Penefsky, H. S. (1973) J. Biol. Chem. 248, 2746–2754
13 Lardy, H., Reed, P. and Chiu Lin, C.-H. (1975) Fed. Proc. 34, 1707–1710
14 Knowles, A. F. and Penefsky, H. S. (1972) J. Biol. Chem. 247, 6617–6623

15 Harris, D. A., Rosing, J., Van de Stadt, R. J. and Slater, E. C. (1973) Biochim. Biophys. Acta 314, 149–153
16 Harris, D. A., Radda, G. K. and Slater, E. C. (1977) Biochim. Biophys. Acta 459, 560–572
17 Bertina, R. M. (1972) The interaction of oligomycin and aurovertin with the ATPase complex in intact mitochondria, Ph.D. thesis, Amsterdam, Gerja, Waarland
18 Penefsky, H. S. and Warner, R. C. (1965) J. Biol. Chem. 240, 4694–4702
19 Garrett, N. E. and Penefsky, H. S. (1975) J. Biol. Chem. 250, 6640–6647
20 Senior, A. E. and Brooks, J. C. (1970) Arch. Biochem. Biophys. 140, 257–266
21 Senior, A. E. (1973) Biochemistry 12, 3622–3626
22 Rosing, J., Harris, D. A., Slater, E. C. and Kemp Jr., A. (1975) J. Supramol. Struct. 3, 284–296
23 Knowles, A. F. and Penefsky, H. S. (1972) J. Biol. Chem. 247, 6624–6630
24 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–766
25 Lowry, O. H., Rosebrough, A. L., Farr, L. H. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
26 Foster, J. F. and Sterman, M. D. (1956) J. Am. Chem. Soc. 78, 3656–3660
27 Yeates, R. A. (1974) Biochim. Biophys. Acta 333, 173–179
28 Baldwin, C. L., Weaver, L. C., Brooker, R. M., Jacobsen, T. N., Osborne, Jr., C. E. and Nash, H. A. (1964) Lloydia 27, 88–95
29 Mulheirn, J. L., Beechey, R. B., Leworthy, D. P. and Osselton, M. D. (1974) J. C. S. Chem. Commun. 874–876
30 Brocklehurst, J. R., Freedman, R. B., Hancock, D. J. and Radda, G. K. (1970) Biochem. J. 116, 721–731
31 Forrest, G. and Edelstein, S. J. (1970) J. Biol. Chem. 245, 6468–6470
32 Lambeth, D. O., Lardy, H. A., Senior, A. E. and Brooks, J. C. (1971) FEBS Lett. 17, 330–332
33 Verschoor, G. J., van der Sluis, P. R. and Slater, E. C. (1977) Biochim. Biophys. Acta 462, 438–449
34 Vadineanu, A., Berden, J. A. and Slater, E. C. (1976) Biochim. Biophys. Acta 449, 468–479
35 Ferguson, S. J., Lloyd, W. J., Radda, G. K. (1976) Biochem. J. 159, 347–353