Biochimica et Biophysica Acta, 462 (1977) 438–449

BBA 47394

THE BINDING OF AUROVERTIN TO ISOLATED β SUBUNIT OF F_1 (MITOCHONDRIAL ATPase)

STOICHEIOMETRY OF β SUBUNIT IN F_1

GERRIT J. VERSCHOOR, PETER R. VAN DER SLUIS and E. C. SLATER

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

(Received April 6th, 1977)

SUMMARY

1. Beef-heart mitochondrial ATPase (F_1) is inactivated and dissociated by incubation with 0.85 M LiCl. ATP partly protects against inactivation. Three dissociation products could be identified after chromatography on diethylaminoethylcellulose: the δ subunit which is not adsorbed, the β subunit which may be eluted from the column, and the α and γ subunits which remain bound to the column.

2. Aurovertin binds to dissociated F_1 with a fluorescence enhancement equal to about 30 % that found with F_1. Unlike intact F_1 which shows two kinetically separated phases of fluorescence enhancement, only a fast phase is found with dissociated enzyme.

3. Fluorescence measurements at varying aurovertin and protein concentrations indicate that aurovertin binds to dissociated F_1 in a simple 3-component reaction with dissociation constant 0.4 μM. There are two indistinguishable binding sites, calculated on the basis of the initial F_1 concentration before dissociation.

4. The β subunit was isolated from dissociated F_1 by DEAE-cellulose chromatography. It has no ATPase activity but reacts with aurovertin with a fluorescence enhancement similar to that of dissociated F_1.

5. The isolated β subunit contains one aurovertin binding site with a dissociation constant of 0.56 μM.

6. It is concluded that F_1 contains two β subunits.

INTRODUCTION

The subunit composition of mitochondrial ATPase (F_1) and related enzymes in other energy-transducing membranes (chloroplasts and bacterial protoplasmic membranes) is still somewhat controversial. Based on the staining intensities of the bands obtained in dodecyl sulphate-polyacrylamide gel electrophoresis, Senior [1] and Catterall et al. [2] proposed the subunit structure $\alpha_3\beta_3\gamma\delta\varepsilon$ for the beef-heart and rat-liver F_1, respectively. This structure has also been proposed for F_1 of *Escherichia coli* [3] and of the thermophilic bacterium P.S. 3 [4], on the basis both of relative staining

in the gels and of incorporation of [^{14}C]amino acids included in the growth medium. Vogel and Steinhart [5] have, however, proposed a dimeric structure $\alpha_2\beta_2\gamma_2\delta_2\varepsilon_2$ for *E. coli* F_1, based on reconstitution of partly dissociated enzyme. In the case of the chloroplast both the relative staining intensities and the ^{14}C contents of the bands obtained by gel electrophoresis in dodecyl sulphate of F_1 isolated from the chloroplasts of peas grown in an atmosphere of [^{14}C]CO_2 indicate a subunit structure of $\alpha_2\beta_2\gamma\delta\varepsilon_2$ [6]. On the basis of labelling of -SH groups with radioactively labelled *N*-ethylmaleimide, Senior has proposed that the heart enzyme contains 2 α, 2 γ and 2 ε subunits [7]. The stoicheiometry of β and δ subunits could not be determined by this method.

The recent report by Wagenvoord et al. [8] that the photo-affinity label 8-azido-ATP is bound specifically to the β subunit of beef-heart F_1 and that complete inactivation after photo-labelling corresponds with the binding of 2 molecules suggests that this enzyme contains 2 β subunits. However, it could not be excluded from these experiments that 3 β subunits are present in intact F_1 and that reaction of only 2 of them causes inactivation of enzyme activity.

In this paper, we show that aurovertin binds to the isolated β subunit and that there are two binding sites for aurovertin in F_1 dissociated by treatment with 0.85 M LiCl. Since intact F_1 also contains two non-distinguishable aurovertin-binding sites [9], it follows that the intact enzyme must contain two β subunits.

METHODS

F_1 was isolated by the method of Knowles and Penefsky [10] and kept in 250 mM sucrose/10 mM Tris/acetate buffer/2 mM EDTA/4 mM ATP (final pH 7.5) at the temperature of liquid nitrogen. After thawing, the enzyme was precipitated by addition of satd. $(NH_4)_2SO_4$ to 55 % satn., and collected by centrifugation.

Dissociated F_1 was prepared by dissolving 10–20 mg protein of the F_1 precipitate in 250 mM sucrose/10 mM Tris · HCl buffer/2 mM EDTA/1 mM ATP/1 mM dithiothreitol/0.85 M LiCl (final pH 8.3) to a final concentration of about 6 mg protein/ml. After 3–4 h at 20 °C, the solution was brought on a Sephadex G-50 column (8 × 1 cm), equilibrated with a buffer containing 250 mM sucrose, 10 mM Tris · HCl buffer, 2 mM EDTA and 1 mM dithiothreitol (final pH 8.3). The column was eluted with this buffer and the fraction containing the protein was either used immediately for preparation of the β subunit or stored in liquid nitrogen.

The β subunit was isolated from the solution of dissociated F_1 by bringing 10–20 mg protein on a DEAE-cellulose column (3 × 1 cm) equilibrated with the sucrose/Tris/EDTA/dithiothreitol buffer, followed in sequence by about 6 ml of this buffer, 10 ml of buffer containing 25 mM Na_2SO_4 and 10 ml of buffer containing 250 mM Na_2SO_4. The β subunit is eluted at the highest salt concentration.

The specific ATPase activity was measured with an ATP-regenerating system either by P_i liberation or spectrophotometrically at 340 nm [11, 8].

Protein was determined by the method of Lowry et al. [12], standardized with bovine serum albumin ($A_{279\ nm} = 6.67$ for a 1 % solution [13]).

The molecular weight of F_1 was assumed to be 319 000 [9], and that of the isolated β subunit 50 000 [14].

Electrophoresis on 12 % polyacrylamide gels in the presence of 0.1 % sodium dodecyl sulphate was carried out according to Weber et al. [15]. Stacking gels were

prepared as described by Mauer [16].

Electrophoresis on 5 % polyacrylamide gels with or without 8 M urea was carried out as described by Knowles and Penefsky [10].

Fluorescence measurements were made with an Eppendorf fluorimeter equipped with a stirring compartment. The excitation filter transmitted at 313 and 366 nm, emission was measured between 470 and 3000 nm.

Aurovertin D isolated by Bertina [17] was used. An absorbance coefficient of $A_{367.5\ nm} = 28.5\ mM^{-1} \cdot cm^{-1}$ was assumed [9].

DEAE-cellulose, type DEAE-23SH and Sephadex G-50 were obtained from Serva and Farmacia, respectively.

RESULTS

Dissociation of F_1 by treatment with LiCl

Beef-heart F_1 is rapidly inactivated by incubation in 0.85 M LiCl at 20–22 °C, the rate of inactivation being higher at low F_1 concentrations (Fig. 1). ATP partly protects against this inactivation (Fig. 2).

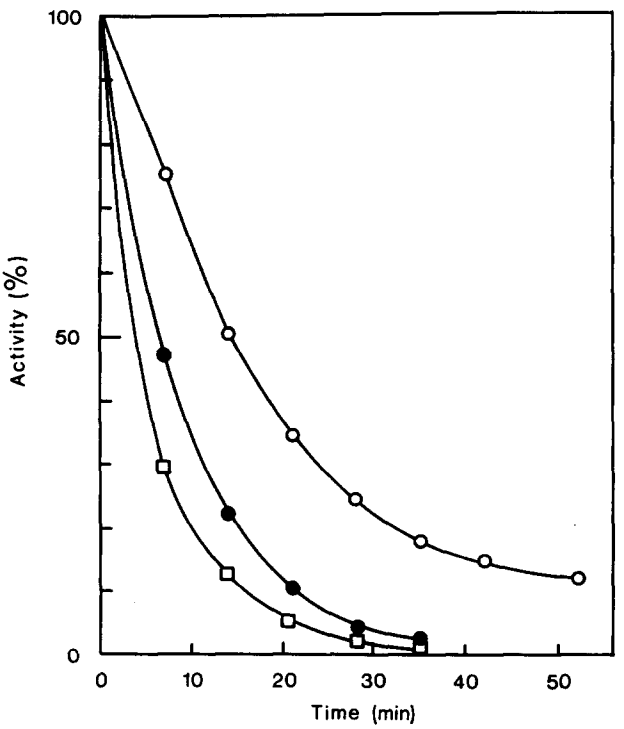


Fig. 1. Effect of protein concentration on inactivation of F_1 by 0.85 M LiCl. An $(NH_4)SO_4$ precipitate of F_1 was dissolved in 250 mM sucrose/10 mM Tris · HCl buffer/2 mM EDTA/1 mM ATP/1 mM dithiothreitol/0.85 M LiCl (final pH, 8.3) at 22 °C. Protein concentration was 8.2 mg/ml (○—○), 4.1 mg/ml (●—●) and 0.1 mg/ml (□—□). Samples were taken at the times indicated and the ATPase activity measured spectrophotometrically. 100 % activity corresponds to a specific activity of 80 μmol · $min^{-1} \cdot mg^{-1}$.

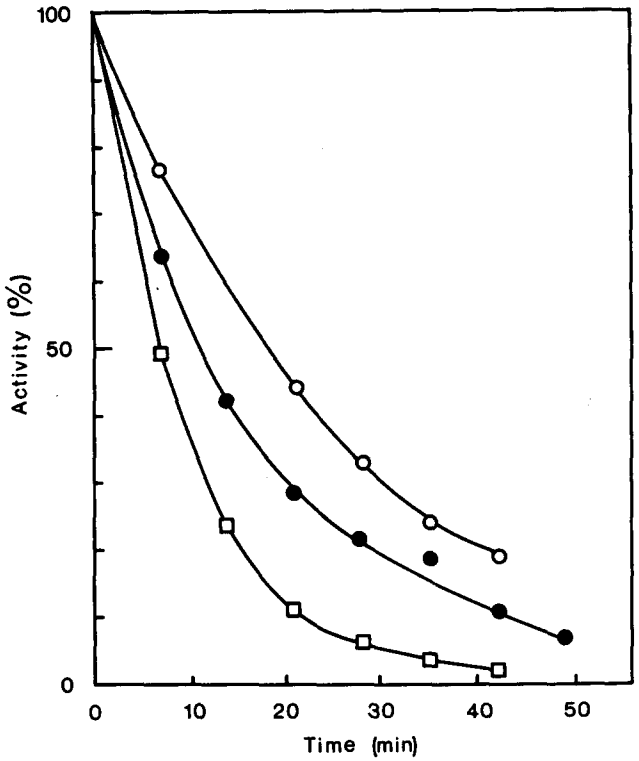


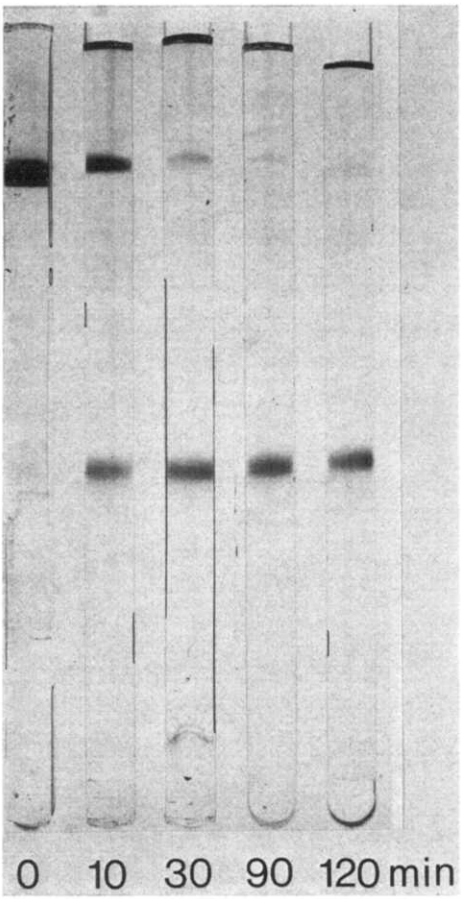
Fig. 2. Effect of ATP concentration on inactivation of F_1 by 0.85 M LiCl. An $(NH_4)_2SO_4$ precipitate of F_1 was dissolved in the same medium as in Fig. 1, except that no (□—□), 1 mM ATP (●—●) or 10 mM ATP (○—○) was present. The F_1 concentration was 5.4 mg/ml. ATPase activity was measured as in Fig. 1.

Polyacrylamide-gel electrophoresis in the absence of dodecyl sulphate shows the appearance during LiCl treatment of a band with $R_F = 0.55$, coincident with the disappearance of the band due to intact F_1 with $R_F = 0.16$ (see Fig. 3). The more mobile band was also obtained with cold-inactivated F_1 applied to 5 % polyacrylamide gels, as previously reported by Knowles and Penefsky [10]. The protein in the mobile band obtained after LiCl treatment or cold inactivation of F_1 co-migrated with the β subunit on 5 % polyacrylamide gels without dodecyl sulphate (see Fig. 4).

Binding of aurovertin to dissociated F_1

Aurovertin binds to dissociated F_1, as to the intact enzyme, with an enhancement of its fluorescence, but the kinetics of the fluorescence enhancement are different from those with the intact enzyme. Whereas two phases are clearly seen with the latter [18, 19], only a fast phase is found with the dissociated enzyme (see Fig. 5). Furthermore, the fluorescence yield of aurovertin bound to dissociated F_1 is considerably less than that bound to the intact enzyme (about 30 %).

Fig. 6 shows a titration of dissociated F_1 with aurovertin at different protein concentrations. The fluorescence yield of bound aurovertin was calculated by the



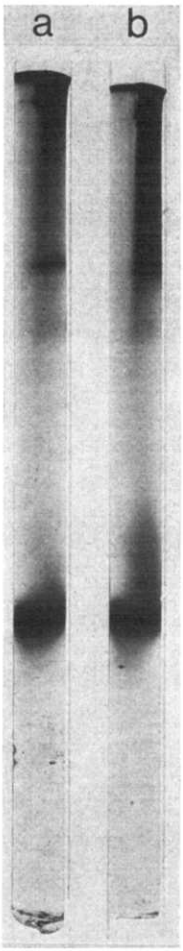


Fig. 3. Electrophoresis in 5 % polyacrylamide gels, in absence of dodecyl sulphate or urea, of F_1 dissociated by 0.85 M LiCl. An $(NH_4)_2SO_4$ precipitate of F_1 was dissolved in the same medium as described in Fig. 1 at 20 °C. The concentration was 2.5 mg/ml. After incubation for the indicated periods 55 μl of the solution was mixed with 10 μl of a 0.02 % bromophenol blue solution and 55 μl of a buffer containing 0.5 M sucrose/100 mM Tris · HCl buffer/4 mM EDTA/8 mM ATP (final pH 7.8). 100 μl of the mixture were layered on a gel prepared as described in Methods.

Fig. 4. Co-electrophoresis of the β subunit with LiCl-treated and cold-inactivated F_1 in 5 % polyacrylamide gels in the absence of dodecyl sulphate or urea. I. Cold-inactivated F_1 was prepared by dissolving an $(NH_4)_2SO_4$ precipitate of F_1 in a buffer containing 10 mM Tris · HCl buffer/2 mM EDTA/50 mM KNO_3 (final pH 7.5) to a protein concn. of 4 mg/ml and incubating 15 min at 0 °C. The sample (0.55 μl) brought on the right-hand side of gel a, contained 0.65 M sucrose/3 mM Tris · HCl buffer/0.5 mM EDTA/14 mM KNO_3 (final pH 7.5)/0.002 % bromophenol blue and 60 μg cold-inactivated F_1. II. LiCl-inactivated F_1 was prepared by dissolving an $(NH_4)_2SO_4$ precipitate of F_1 in a buffer containing 250 mM sucrose/10 mM Tris · HCl buffer/2 mM EDTA/0.85 M LiCl (final pH 7.5) to a protein concn. of 4 mg/ml and keeping for 5 min at 20 °C. The sample (55 μl) brought on the right-hand side of gel b, contained 0.57 M sucrose/3.5 mM Tris · HCl buffer/0.5 mM EDTA/0.23 M LiCl (final pH 7.5)/0.002 % bromophenol blue and 60 μg of dissociated F_1. III. A sample (55 μl), containing 14 μg β subunit, dissolved in the same solution as I, was brought on to the left-hand side of gel a. IV. A sample (55 μl), containing 14 μg β subunit dissolved in the same solution as II, was

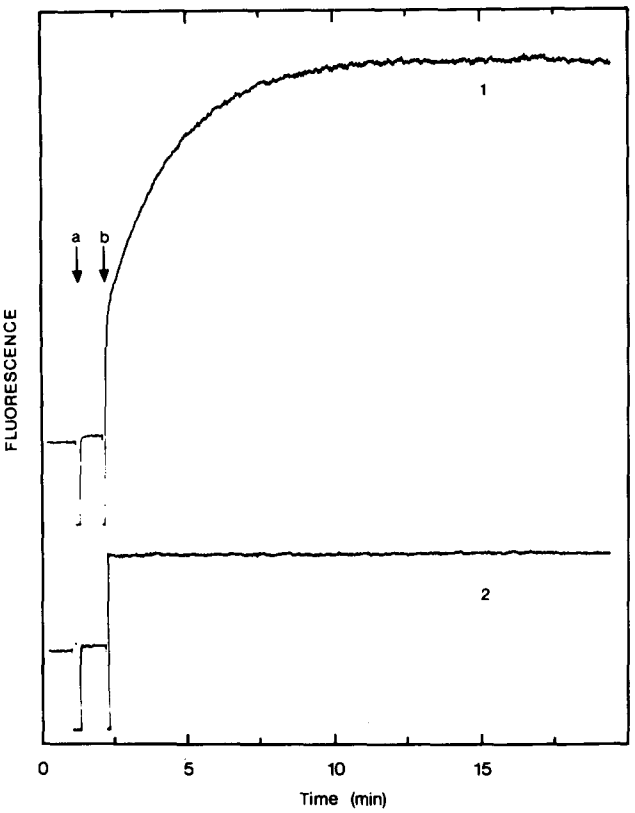


Fig. 5. Kinetics of enhancement of fluorescence on binding of aurovertin to intact and dissociated F_1. Curve 1 shows the fluorescence measured after adding 0.9 μM F_1 (at a) to 250 mM sucrose/10 mM Tris · HCl buffer (pH 8.3), followed, at b, by 0.26 μM aurovertin. Curve 2 shows a similar experiment in which 0.9 μM dissociated F_1 was used. By making use of the binding constants of aurovertin to intact [12] and dissociated (see Table 1 of this paper) F_1, it can be calculated that 93 % of the aurovertin was bound to intact F_1 and 80 % to dissociated F_1. Allowing for the different degree of binding, it can be calculated that the fluorescence of aurovertin bound to intact F_1 is 3.43 times that bound to dissociated F_1.

double-reciprocal plot shown in Fig. 7 (cf. refs. 20 and 9) and the Scatchard plots shown in Fig. 8 were calculated according to Muller et al. [9]. In contrast to the behaviour with the intact enzyme [9], straight Scatchard plots are obtained indicating that the binding is a simple three-component reaction, $E+A \rightleftharpoons EA$. The mean number of binding sites calculated from the Scatchard plots is 1.88 per molecule of undissociated

brought on to the left-hand side of gel b. The procedure described by Knowles and Penefsky [8] was used, except that EDTA and ATP were omitted from the solution that was brought on the gels after the sample, and ATP was omitted from the solution used in preparing the gels. The two samples were kept separate on the gel by a plastic strip.

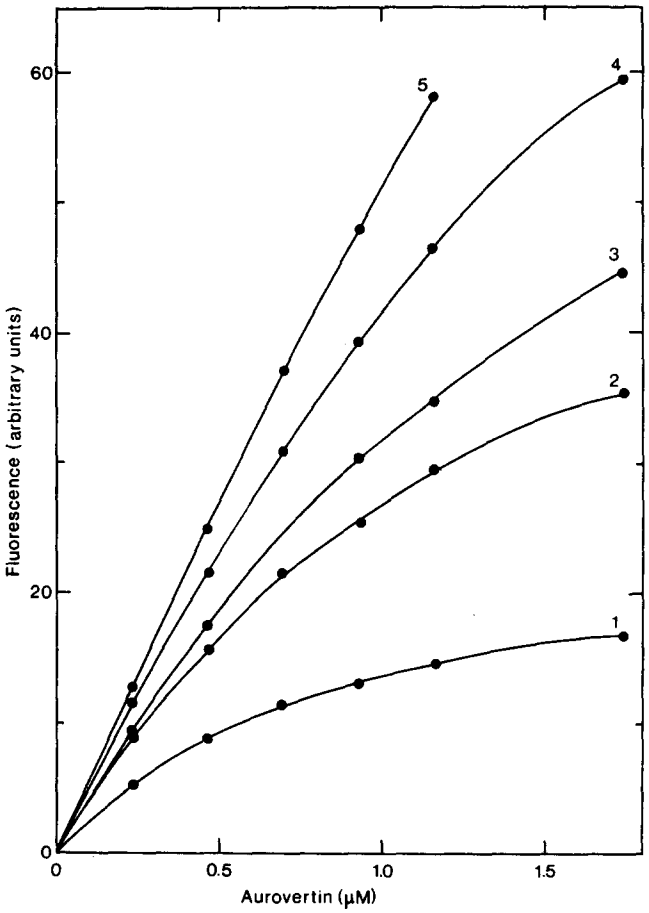


Fig. 6. Titration of dissociated F_1 with aurovertin. Different amounts of aurovertin were added to 0.14 μM (curve 1), 0.35 μM (curve 2), 0.52 μM (curve 3), 0.69 μM (curve 4) and 1.38 μM (curve 5) dissociated F_1 added to 250 mM sucrose/Tris · HCl buffer (pH 8.3) and the fluorescence measured. The final concentrations of EDTA and dithiothreitol, derived from the stock solution of dissociated F_1, varied between 28 μM and 0.28 mM, and 14 μM and 0.14 mM, respectively.

F_1, with a dissociation constant of 0.41 μM (see Table I). The straight Scatchard plot indicates that aurovertin is independently bound to the two sites*.

Binding of aurovertin to isolated β subunit

The β subunit was isolated from dissociated F_1 by DEAE-cellulose chromatography as described in Methods. The δ subunit was detected in the void volume by polyacrylamide-gel electrophoresis in the presence of 8 M urea (gel B3, Fig. 9). According to polyacrylamide-gel electrophoresis in the presence of either 0.1 % sodium dodecyl sulphate or 8 M urea, the fraction eluted with 0.25 M Na_2SO_4 contained only the β subunit (gels A_2, A_3, B_1 and B_2, Fig. 9). The α and γ subunits remain bound to the DEAE-cellulose from which they could be extracted by sodium

* See note added in proof, p. 448.

TABLE I

PARAMETERS OF BINDING OF AUROVERTIN TO DISSOCIATED F_1 AND TO ISOLATED β SUBUNIT

	Dissociated F_1			Isolated β subunit		
	$[F_1]$ (μM)	n*	K_D (μM)	$[\beta]$ (μM)	n**	K_D (μM)
	0.14	1.96	0.39	0.23	1.17	0.55
	0.34	1.91	0.40	0.58	1.04	0.54
	0.52	1.69	0.45	0.81	0.98	0.53
	0.69	1.94	0.42	1.16	1.07	0.53
				2.33	0.95	0.60
				2.91	0.97	0.59
Mean		1.88	0.41		1.03	0.56

* Number of binding sites per 319 000 daltons protein.
** Number of binding sites per 50 000 daltons protein.

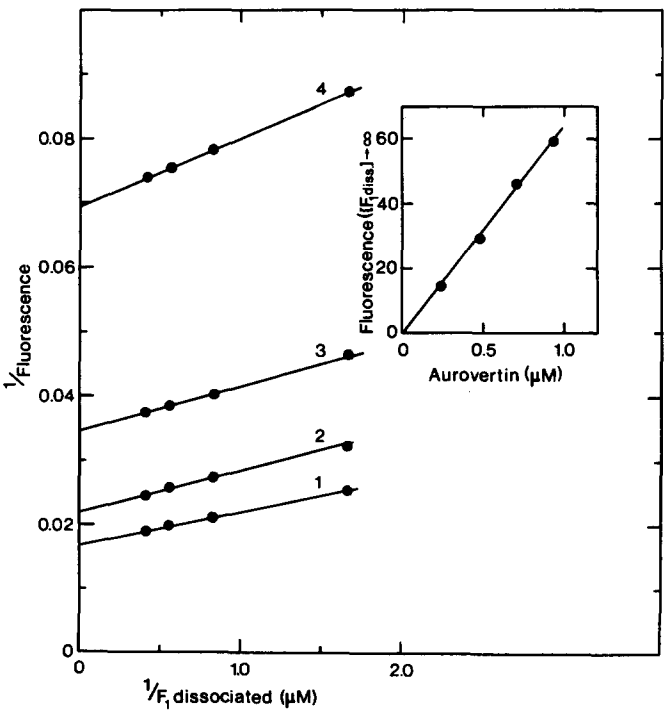


Fig. 7. Double-reciprocal plot of fluorescence against concentration of dissociated F_1 at 0.93 (1), 0.70 (2), 0.46 (3) and 0.23 μM (4) aurovertin, respectively. Reaction conditions as described in Fig. 6. The insert shows a plot of fluorescence at infinite protein concentration against aurovertin concentration. From the slope of this line, it can be calculated that 1 μM aurovertin bound to the protein yields a fluorescence of 64.5 units.

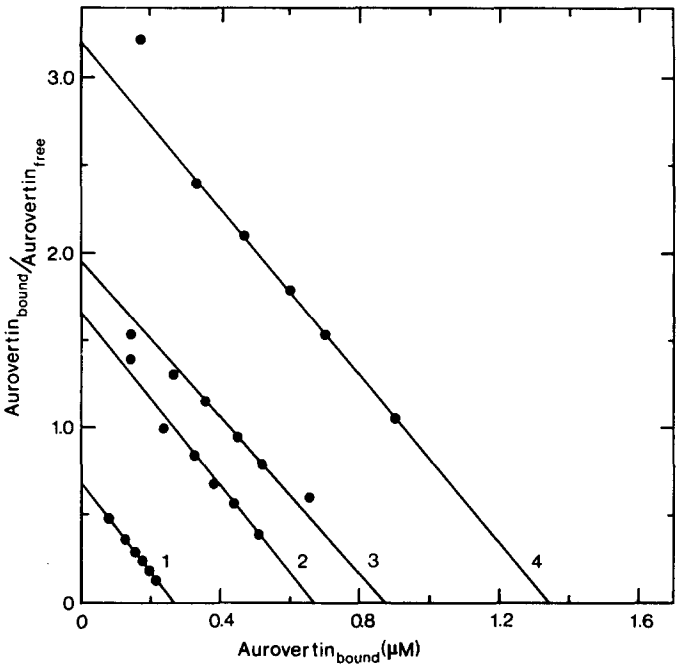


Fig. 8. Scatchard plot of data shown in Fig. 6 for 0.14 (1), 0.35 (2), 0.52 (3) and 0.69 μM (4) dissociated F_1, using the value for the fluorescence of F_1-bound aurovertin calculated in Fig. 7.

dodecyl sulphate or urea. Owing to its low concentration, it was not possible to determine whether the ε subunit remains in the column or appears in the void volume. It is not present in the 0.25 M Na_2SO_4 eluate.

The isolated β subunit has no detectable ATPase activity ($<$ 1 nmol P_i/mg protein per min). However, like the β subunit in the intact F_1 [8], the isolated β subunit reacts with 8-azido-ATP. Aurovertin also binds with the isolated β subunit with a fluorescence enhancement of 27-fold, compared with 32-fold for dissociated F_1. Binding parameters were calculated from the Scatchard plots (Fig. 10), obtained in exactly the same way as for dissociated F_1. Table I shows that the isolated β subunit binds 1 molecule of aurovertin with a dissociation constant of 0.56 μM.

DISCUSSION

Beef-heart F_1 is dissociated by treatment with 0.85 M LiCl at 20 °C into at least 3 components - the β subunit, the δ subunit and the rest of the molecule - which may be separated by chromatography on DEAE-cellulose. The enzymic activity is also lost after this treatment, and is not restored by desalting. In this respect, the beef-heart enzyme differs from *E. coli* F_1 which is stable to 1 M LiCl at room temperature, and is inactivated only after freezing at −70 °C in the presence of 1 M LiCl [5] and is reactivated by desalting. In fact, the differences between the mammalian and bacterial enzyme are perhaps greater than is generally assumed. Although both enzymes as isolated contain 5 subunits, the ε subunit of the bacterial enzyme [21] corresponds to

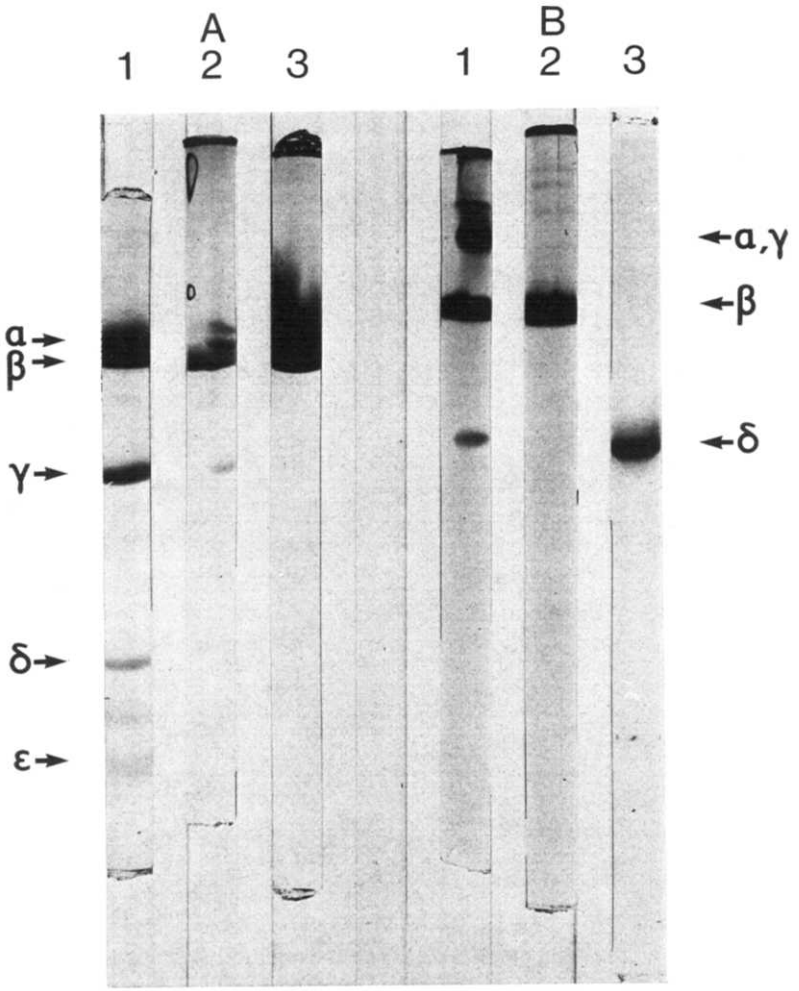


Fig. 9. Polyacrylamide-gel electrophoresis of F_1 and of its subunits. The gels shown in A are 10 % polyacrylamide, containing 0.1 % dodecyl sulphate. A1, 50 μg F_1; A2, on the left 4 μg β subunit, on the right 7 μg F_1; A3, 100 μg β subunit. The gels shown in B are 5 % polyacrylamide gels containing 8 M urea. B1, on the left 25 μg β subunit and on the right 75 μg of F_1. The ε subunit that, according to Knowles and Penefsky [8] is found between the band containing α and γ, and the band containing β, was not detected in our experiments. B2, 50 μg β subunit; B3, a concentrated fraction of the void volume of the DEAE-cellulose column.

the inhibitor of the heart enzyme which is a sixth subunit of that enzyme [22]. Furthermore, no OSCP (oligomycin-sensitivity conferring protein) [23] has been detected in bacteria and it is possible that the δ subunit of the bacterial enzyme takes over its role [24]. Since addition of inhibitor [25] stabilizes F_1 to cold inactivation, the different subunit composition of the isolated heart and bacterial enzymes may explain the different stabilities.

Since (i) dissociated F_1 contains two identical aurovertin-binding sites, cal-

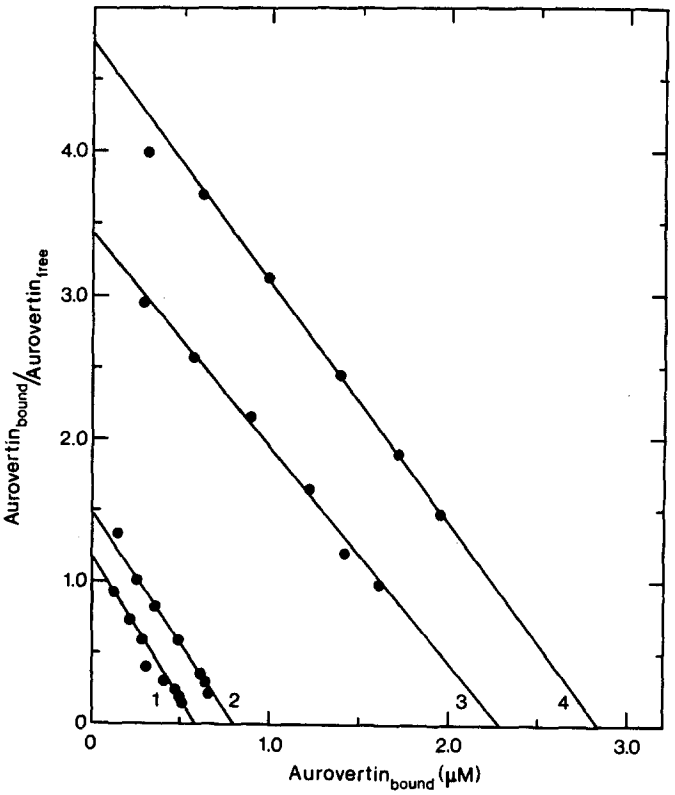


Fig. 10. Scatchard plots derived from an aurovertin titration of the isolated β subunit carried out in the same way as for dissociated F_1, as shown in Figs. 6–8. Concentration of β subunit: 1, 0.58 μM; 2, 0.82 μM; 3, 2.33 μM; 4, 2.91 μM.

culated on basis of the initial F_1 concentration and (ii) the isolated β subunit contains one aurovertin-binding site with a similar dissociation constant, it may be concluded that F_1 contains two β subunits. Both units in intact F_1 react with aurovertin [9] and 8-azido-ATP [8].

Taken in conjugation with Senior's [7] data on the binding of *N*-ethylmaleimide, it follows that F_1 has the subunit structure $\alpha_2\beta_2\gamma_2\delta_x\varepsilon_2$. If x is also equal to 2, a protein molecular weight of 324 000 may be calculated, which is in good agreement with the ultracentrifugation data, corrected for the 8 % non-protein material present [9].

NOTE ADDED IN PROOF (Received September 15, 1977)

Since this manuscript was submitted, additional experiments similar to those summarized in Table I have been carried out in which LiCl was not removed after dissociation of F_1 and fluorescence was measured in the presence of 0.85 M LiCl. The mean number of aurovertin-binding sites was calculated to be 2.06 per molecule of undissociated F_1.

ACKNOWLEDGEMENTS

We wish to thank Dr. J. Rosing, Mr. J. L. M. Muller and Dr. A. Kemp for stimulating discussions. This research was supported by a grant from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.), under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and by NATO Research Grant No. 563 to Dr. G. K. Radda and one of us (E.C.S.).

REFERENCES

1 Senior, A. E. (1973) Biochim. Biophys. Acta 301, 249–277
2 Catterall, W. A., Coty, W. A. and Pedersen, P. L. (1973) J. Biol. Chem. 248, 7427–7431
3 Bragg, P. D. and Hou, C. (1975) Arch. Biochem. Biophys. 167, 311–321
4 Kagawa, Y., Sone, N., Yoshida, M., Hirata, H. and Okamoto, H. (1976) J. Biochem. 80, 141–151
5 Vogel, G. and Steinhart, R. (1976) Biochemistry 15, 208–216
6 Nelson, N. (1976) Biochim. Biophys. Acta 456, 314–338
7 Senior, A. E. (1975) Biochemistry 14, 660–664
8 Wagenvoord, R. J., Van der Kraan, I. and Kemp, A. (1977) Biochim. Biophys. Acta, 460, 17–24
9 Muller, J. L. M., Rosing, J. and Slater, E. C. (1977) Biochim. Biophys. Acta 462, 422–437
10 Knowles, A. F. and Penefsky, H. S. (1972) J. Biol. Chem. 247, 6617–6623
11 Pullman, M. E., Penefsky, H. S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
12 Lowry, O. H., Rosebrough, A. L., Farr, L. H. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
13 Foster, J. F. and Sterman, M. D. (1956) J. Am. Chem. Soc. 78, 3656–3660
14 Knowles, A. F. and Penefsky, H. S. (1972) J. Biol. Chem. 247, 6624–6630
15 Weber, K., Pringle, J. R. and Osborn, M. (1972) in Methods in Enzymology, Vol. XXVI (Colowick, S. P. and Kaplan, N. O., eds), pp. 3–27, Academic Press, New York
16 Maurer, H. R. (1971) Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis, W. de Gruyter, Berlin
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 Yeates, R. A. (1974) Biochim. Biophys. Acta 333, 173–179
19 Chang, T. M. and Penefsky, H. S. (1973) J. Biol. Chem. 248, 2746–2754
20 Brocklehurst, J. R., Freedman, R. B., Hancock, D. J. and Radda, G. K. (1970) Biochem. J. 116, 721–731
21 Nieuwenhuis, F. J. R. M. and Bakkenist, A. R. J. (1977) Biochim. Biophys. Acta 459, 596–604
22 Brooks, J. C. and Senior, A. E. (1971) Arch. Biochem. Biophys. 147, 467–470
23 MacLennan, D. H. and Tzagoloff, A. (1968) Biochemistry 7, 1603–1610
24 Futai, M., Sternweis, P. C. and Heppel, L. A. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2725–2729
25 Pullman, M. E. and Monroy, G. C. (1963) J. Biol. Chem. 238, 3762–3769