Chemical Modification of F_1-ATPase by Dicyclohexylcarbodiimide: Application to Analysis of the Stoichiometry of Subunits in *Escherichia coli* F_1†

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ABSTRACT: *N,N'*-Dicyclohexylcarbodiimide (DCCD) covalently binds to the β subunit of *Escherichia coli* F_1-ATPase (BF_1). The ATPase activity is fully inhibited when 1 mol of DCCD is bound/mol of BF_1, in spite of the fact that BF_1 contains several β subunits [Satre, M., Lunardi, J., Pougeois, R., & Vignais, P. V. (1979) *Biochemistry 18*, 3134–3140]. Advantage was taken of the reactivity of DCCD with respect to BF_1 to determine the exact stoichiometry of the β subunits in BF_1. Two methods were used. The first one was based on the fact that modification of the β subunit by DCCD results in the disappearance of one negative charge, due to the binding of DCCD to a carboxyl group of the β subunit. The nonmodified and the modified β subunits were separated by electrofocusing, and the percentage of modified β subunits was assessed as a function of the percentage of ATPase inactivation. The second method relied on direct comparison, after inactivation of BF_1 by [^{14}C]DCCD, of the specific radioactivities of the whole BF_1 and the isolated β subunits. Both methods indicate that each molecule of BF_1 contains three β subunits.

The catalytic sector, F_1, of the H^+-linked ATPases isolated from mitochondria, bacteria, and chloroplasts is characterized by large structural similarities. Whatever the source of F_1, the M_r value found is in the range of 320 000–380 000; each molecule of F_1 contains five different types of subunits, named α, β, γ, δ, and ϵ with M_r of 62 000–57 000 for α, 56 000–50 000 for β, 37 000–31 000 for γ, 18 000–15 000 for δ, and 13 000–5000 for ϵ [for a review, see Futai & Kanazawa (1980), Kagawa et al. (1980), and Dunn & Heppel (1981)]. It is clear that multiple copies of each subunit are present in each molecule of F_1. However, the stoichiometry of these subunits remains a matter of debate, in spite of many experiments based on a number of approaches, including quantitative analysis by staining of F_1 subunits isolated by sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis (Catterall et al., 1973; Takeshige et al., 1976; Binder et al., 1978), biosynthetic incorporation of ^{14}C-labeled amino acids in F_1 (Bragg & Hou, 1975; Nelson, 1976; Kagawa et al., 1976; Huberman & Salton, 1979; Stutterheim et al., 1981), chemical labeling of SH groups in the whole enzyme and the individual subunits (Senior, 1975; Yoshida et al., 1978, 1979; Gregory & Hess, 1981), inactivation of F_1 by 5'-(*p*-fluorosulfonyl-[^{14}C]benzoyl)adenosine (Esch & Allison, 1979), reconstitution of active F_1 from isolated subunits (Vogel & Steinhart, 1976; Dunn & Futai, 1980), electron microscopy of two-dimensional crystals (Wakabayashi et al., 1977), and X-ray diffraction studies (Amzel & Pedersen, 1978; Amzel, 1981). In brief, two types of stoichiometry have been proposed; they are $\alpha_3\beta_3\gamma\delta\epsilon$ and $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$ [for a review, see Futai & Kanazawa (1980)].

The aim of this paper is to present a new approach to the analysis of the F_1 subunits stoichiometry, based on covalent chemical modification. The chemical modifier used here was *N,N'*-dicyclohexylcarbodiimide (DCCD). A useful peculiarity of the inactivation of *Escherichia coli* BF_1 by DCCD, on which our experimental approach is based, is that BF_1 exhibits a partial site reactivity with respect to DCCD; full inactivation is attained when 1 mol of DCCD has bound to 1 mol of β subunit, in spite of the fact that BF_1 contains two or three β subunits (Satre et al., 1979). Modification of the β subunit by DCCD results in the disappearance of one negative charge as shown by electrofocusing; this is due to the binding of DCCD to a carboxyl group (Satre et al., 1979), later identified to the free carboxyl of a glutamyl residue (Esch et al., 1981; Yoshida et al., 1981). Two sets of experiments were carried out in the present work. In the first one, the stoichiometry of the β subunits was calculated from the respective amounts of DCCD-modified β subunits and nonmodified β subunits, after separation of the two types of β subunit by electrofocusing on a polyacrylamide gel. In the second set of experiments, the specific radioactivity of [^{14}C]DCCD-inactivated BF_1 was compared to that of the isolated β subunits, and the stoichiometry of the β subunits was calculated from the ratio of the two values. From both approaches, a stoichiometry of three β subunits per F_1 was found.

Experimental Procedures

Materials. [^{14}C]DCCD (54 mCi/mmol) and [^{14}C]NEM (30 mCi/mmol) in solution in pentane were obtained from the Commissariat à l'Energie Atomique, Saclay, France. After evaporation of pentane under nitrogen, the radioactive compounds were taken up in methanol. The final concentrations of methanol resulting from addition of [^{14}C]DCCD and [^{14}C]NEM solutions were equal to or lower than 1%. Ampholines were purchased from LKB.

Methods. The purification steps of *E. coli* F_1 (BF_1) were the same as those previously described (Satre et al., 1979), including release of the enzyme by chloroform treatment and chromatography on DEAE-cellulose and Sepharose 6B. This BF_1 preparation was virtually devoid of the δ subunit. Calculations were based on a value of 340 000 for the M_r of BF_1 and a M_r of 50 000 for the isolated β subunit. The actual

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DCCD, *N,N'*-dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; Mops, 3-(*N*-morpholino)propanesulfonic acid; BF_1, *E. coli* F_1-ATPase; β-DCCD, β subunit chemically modified by DCCD; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

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molecular weights derived from DNA sequencing data (Sarraste et al., 1981) are 50 157 for the β subunit and 302 050 for $BF_1 = \alpha_2\beta_2\gamma_2\epsilon_2$ or 361 867 for $BF_1 = \alpha_3\beta_3\gamma\epsilon$. The use of 340 000 for the molecular weight of BF_1 is valid within an error of about 10% with respect to these theoretical values. Measurement of ATPase activity was carried at 30 °C by determination of the inorganic phosphate released from ATP (Satre et al., 1979). Protein concentration was determined by the Coomassie blue method of Bradford (1976), with bovine serum albumin as standard; the validity of the dye-binding assay for *E. coli* ATPase and its subunits is documented by the work of Dunn & Futai (1980). Radioactivity was measured by liquid scintillation counting with a toluene–Triton X-100 scintillation fluid (Patterson & Greene, 1965). The yield of counting was determined with a calibrated solution of [^{14}C]toluene obtained from the Laboratoire de Mêtrologie, Saclay, France.

Electrophoresis on 7.5% polyacrylamide gels prepared in 25 mM Tris, 0.19 M glycine, and 0.1% $NaDodSO_4$ was carried out in cylindrical tubes at 2 mA/tube for 3 h. The gels were stained for 4 h in 10% acetic acid, 25% isopropyl alcohol, and 0.05% Coomassie blue R250 and again for 4 h in 10% acetic acid, 10% isopropyl alcohol, and 0.005% Coomassie blue R250. They were then destained in 10% acetic acid with several changes. For determination of the distribution of radioactivity, the gels were frozen in powdered solid CO_2 and cut into 1-mm slices with a Joyce-Loebl instrument. The slices were digested by overnight incubation at 55–60 °C with 1 mL of 15% H_2O_2 in closed scintillation vials. The samples were cooled, and the radioactivity was measured after addition of 10 mL of scintillation fluid.

Chemical modification of BF_1 by DCCD was performed by the addition of 10 μM DCCD (final concentration) to a solution of BF_1 in 25 mM Mops, pH 6.5, at the final concentration of 3 mg/mL; the percentage of methanol in the medium after addition of DCCD was 1%. After incubation for various periods of time at 30 °C, 5-μL samples were diluted 20-fold in 50 mM Tris–SO_4, pH 8.5, to stop inactivation by DCCD, and ATPase activity was measured on small aliquots. Separation of bound and free DCCD was accomplished by filtration through a short column of Sephadex G-50 (fine) equilibrated in 20 mM sodium phosphate, pH 7.4, by the elution–centrifugation method described by Penefsky (1977). Following centrifugation, the DCCD-modified BF_1 and the nonmodified BF_1 were recovered in the eluate.

Labeling of BF_1 by [^{14}C]NEM was carried out by the addition of 1 mM [^{14}C]NEM and incubation for 30 min at 30 °C, followed by the addition of 2% 2-mercaptoethanol to remove the nonreacted [^{14}C]NEM.

Isoelectrofocusing in urea–polyacrylamide gels was performed in glass tubes of 120-mm length and 3-mm inside diameter. The gels were prepared and run as described in detail by O'Farrell (1975) except that Nonidet P-40 was replaced by Triton X-100. They contained 1.6% ampholines, pH 4–6, and 0.4% ampholines, pH 3.5–10. The BF_1 subunits to be subjected to eletrofocusing were dissolved in 9.5 M urea, 2% Triton X-100, a mixture of 1.6% ampholines, pH 4–6, and 0.4% ampholines, pH 3.5–10, 5% 2-mercaptoethanol, and 0.2% $NaDodSO_4$. The amount of protein used per gel was 30 μg. One of the gels was run without protein to measure the pH gradient. After the run, the other gels were stained in 0.1% Coomassie blue R250, 10% trichloroacetic acid, 3% sulfosalicylic acid, and 25% methanol for 1 h at room temperature. They were destained first in 30% ethanol and 10% acetic acid and then in 10% acetic acid. The isoelectric point of the

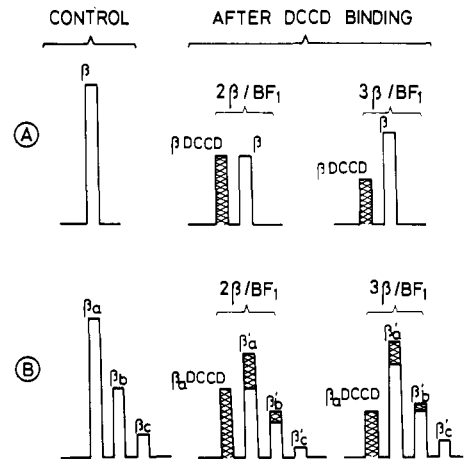

FIGURE 1: (A) Simplified scheme predicting distribution of control β subunits and DCCD-modified β (β-DCCD) subunits after electrofocusing, under conditions of full inactivation of BF_1, i.e., when 1 mol of DCCD has bound to 1 mol of BF_1, for two possible stoichiometries of β subunits. (B) Predicted distribution of control β subunits and β-DCCD subunits, taking into account charge heterogeneity of β subunits. For details, see Results.

nondified β subunit was 5.3 (Satre et al., 1979). For determination of the distribution of the ^{14}C radioactivity in the β subunit region, after labeling by [^{14}C]NEM, the gels were cut in 0.5-mm slices and digested as described above, for scintillation counting.

Results

Stoichiometry of β Subunits in BF_1, As Determined after Electrofocusing of DCCD-Modified and Nonmodified β Subunits. (1) Theory. It was previously shown that DCCD-modified β subunits (β-DCCD subunits) and nonmodified β subunits could be separated by electrofocusing; this was possible because the binding of DCCD to a carboxyl group of the β subunit led to the concomitant decrease of one negative charge. On the basis of the following facts (Satre et al., 1979), a simple scheme (Figure 1A) was drawn to predict the percentage of β-DCCD subunits that could be expected to accumulate under conditions of full inactivation, i.e., when 1 mol of DCCD has bound to 1 mol of BF_1: (1) DCCD binds specifically to the β subunit of BF_1, (2) DCCD binding is linearly related to inactivation of BF_1, and (3) full inactivation corresponds to an extrapolated values of 1 mol of bound DCCD/mol of BF_1. In this scheme, two possible stoichiometries were envisaged, namely, two and three β subunits in BF_1. However, the situation was more complicated in practice, because of the charge heterogeneity of β subunits even in freshly purified BF_1. Charge heterogeneity is a widespread phenomenon in proteins, due essentially to spontaneous deamidation of unstable glutaminyl and asparaginyl residues (O'Farrell, 1975). This heterogeneity is detected on electrofocusing in gels. It results in the formation of several discrete bands, separated from each other by short and equal spacings, consistent with single charge differences between the bands; the more acidic bands are less intense. In the specific case of the β subunit of freshly prepared BF_1, instead of a single band, electrofocusing revealed at least three equally spaced bands. One of them was predominant; it most likely corresponded to the native β subunit and was referred to as β_a. The two other bands (satellite bands) were in minor amounts and had a more acidic isoelectric point than β_a; they were designated β_b and β_c in order of decreasing abundance and increasing acidity. Other satellite bands were hardly detectable and were therefore neglected. The native β_a band and the β_b

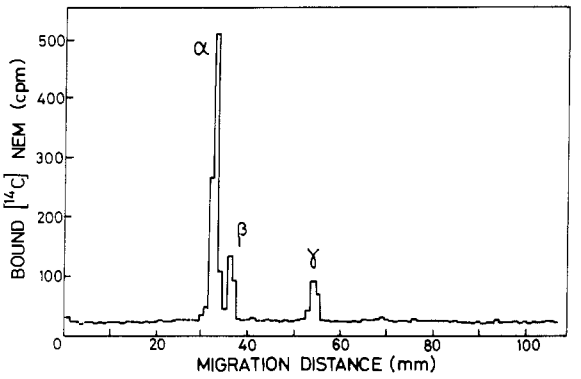

FIGURE 2: Labeling of BF_1 subunits with [^{14}C]NEM. BF_1 (0.36 mg/mL) was incubated for 30 min with 1% $NaDodSO_4$ and 1 mM [^{14}C]NEM. The incubation was stopped by the addition of 2% 2-mercaptoethanol, and the sample (0.1 mL) was subjected to centrifugation–filtration through a Sephadex G-50 (fine) column equilibrated in 25 mM Tris, 0.19 M glycine, and 0.1% $NaDodSO_4$, pH 8.5. The eluate was supplemented with glycerol (20% final concentration), and a portion (about 10 μg of protein) was subjected to electrophoresis (cf. Methods).

and β_c satellites could be identified by Coomassie blue staining; however, a better way to trace them, due to the small amount of β_b and β_c, was by [^{14}C]NEM labeling as detailed thereafter. The equal spacing between β_a, β_b, and β_c and the higher acidity of β_b and β_c was explained, in agreement with the proposal of O'Farrell (1975), by assuming that β_b and β_c were derived from β_a by release of amino groups. Charge heterogeneity of β subunits was taken into account in the scheme of Figure 1B to predict the distribution of the β-DCCD subunits after electrofocusing, under conditions of full inactivation of BF_1. Here again the two possible stoichiometries, namely, two and three β subunits per BF_1, were envisaged. The scheme shows that the binding of 1 mol of DCCD to 1 mol of β_a subunit results in β_a-DCCD that is characterized by a more basic isoelectric point than β_a. Binding of DCCD to β_b and β_c should also result in derivatives with a more alkaline isoelectric point than the original peptides; consequently, the β_b-DCCD subunit should migrate like β_a and the β_c-DCCD subunit like β_b.

In practice, the nonmodified β subunits and the β-DCCD subunits were separated from each other by electrofocusing. For identification of the bands, the β subunit was labeled by [^{14}C]NEM (cf. Methods). This is in fact a feature of the β subunit of BF_1 to be labeled by [^{14}C]NEM, in agreement with the occurrence of one cysteine residue in the β subunit (Sarraste et al., 1981), which contrasts with the absence of labeling in the β subunit of mitochondrial F_1 (Senior, 1975). The labeling pattern of BF_1 by [^{14}C]NEM is shown in Figure 2. The labeling ratio in the α and β subunits, calculated from four experiments, ranged from 2.7 to 4.3. An obvious advantage of labeling by [^{14}C]NEM is that the net charge of the β subunit is not disturbed.

Figure 3 shows the radioactivity profile of the β-subunit region of a gel after electrofocusing of (1) control BF_1 labeled by [^{14}C]NEM and (2) DCCD-modified BF_1 postlabeled by [^{14}C]NEM. The main β subunit, called β_a, the two satellite β subunits β_b and β_c, and the supplementary band corresponding to β_a modified by DCCD were easily identified by [^{14}C]NEM labeling. From the labeling data, following the electrofocusing step, the stoichiometry of the β subunits in BF_1 was derived as follows. First, in control BF_1 that had not been exposed to DCCD we calculated the proportion, x, of subunit β_a relative to the sum of all β subunits, i.e., β_a and the satellite subunits β_b and β_c:

$$x = \beta_a/(\beta_a + \beta_b + \beta_c) \quad (1)$$

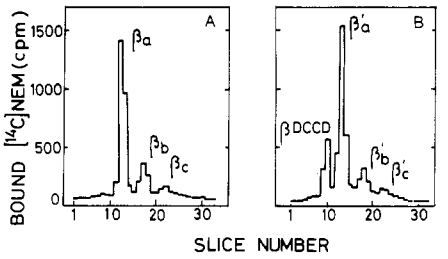

FIGURE 3: Radioactivity profile of β-subunit region after labeling with [^{14}C]NEM and electrofocusing. Either control BF_1 (panel A) or BF_1 inactivated with DCCD (panel B, 68% inactivation; see Table I) was labeled with [^{14}C]NEM and subjected to electrofocusing as described under Methods.

Table I: Calculation of the Number of β Subunits in BF_1 after Inactivation with DCCD and Separation of β-DCCD Subunits from Nonmodified β Subunits by Isoelectrofocusing

BF_1 preparations	ATPase inactivation (%)	y = β_a-DCCD/$\Sigma\beta$	no. of β subunits per BF_1
I[a]	39	0.061	3.4
	61	0.099	3.3
	65	0.11	3.2
	78	0.16	2.6
	83	0.16	2.8
II[a]	42	0.090	3.0
	59	0.13	2.9
	68[b]	0.16[b]	2.7
	85	0.18	3.0

[a] Data were collected for two independent preparations of BF_1 characterized by different levels of native β (β_a). In set I, x, i.e., $\beta_a/(\beta_a + \beta_b + \beta_c)$, was equal to 0.53 and in set II to 0.64. Aliquots of BF_1 were inactivated with DCCD to various extents before labeling with [^{14}C]NEM and isoelectrofocusing. For details and further calculations see the text. [b] Experimental data shown in Figure 3.

Second, with DCCD-inactivated BF_1, the proportion of β_a-DCCD subunits was given by

$$y = \beta_a\text{-DCCD}/(\beta_a\text{-DCCD} + \beta_a' + \beta_b' + \beta_c') \quad (2)$$

β_a' corresponded to the sum of nonmodified β_a and β_b-DCCD, and so forth for β_b' and β_c'. Third, for a given percentage of inactivation, i, n being the number of β subunits per molecule of BF_1, the relation between y, x, i, and the number n of β subunits in BF_1 could be writted as

$$y = (i/100)(x/n) \quad (3)$$

It must be recalled that the later relation relies on the fact that inactivation of BF_1 is directly proportional to the extent of inactivation and that full inactivation (i = 100%) corresponds to an extrapolated binding of 1 mol of DCCD/mol of BF_1.

(*2*) *Experimental Data.* Labeling of BF_1 by DCCD was carried out as described under Methods. This was followed by labeling by [^{14}C]NEM (cf. Methods). The nonmodified β subunits and the β-DCCD subunits were separated from each other by electrofocusing. The gels were sliced, and the radioactivity of the slices was counted. The data obtained from the profiles of radioactivity were used in the above equations. The stoichiometry of the β subunits in BF_1, derived from eq 3, was found to be 2.7 in the specific case of the experiment illustrated in Figure 3. In Table I, are collected the data on stoichiometry, corresponding to two preparations of BF_1 and nine different assays in which inactivation by DCCD ranged from 39% to 85%. The n value was equal to 3.0 ± 0.3 (SEM), indicating that there are three β subunits per molecule of BF_1.

Stoichiometry of β Subunits in BF_1, As Determined by Correlation between Extent of Radiolabeling by [^{14}C]DCCD

Table II: Inactivation of BF_1 by [^{14}C]DCCD: Comparison of the Specific Radioactivity of BF_1 and of Isolated β Subunits

ATPase inactivation[a] (%)	[^{14}C]-DCCD/BF_1[a] (mol/mol)	[^{14}C]-DCCD/BF_1[b] (mol/mol)	[^{14}C]-DCCD/β (mol/mol)	no. of β subunits per BF_1[c]
74	0.79	0.76	0.25	3.0[d]
32	0.36	0.49	0.17	2.9
37	0.38	0.36	0.14	2.6
55	0.56	0.68	0.24	2.8
55	0.52	0.64	0.27	2.4
67	0.90	0.82	0.26	3.2
92	0.91	0.95	0.36	2.6

[a] ATPase activity and [^{14}C]DCCD incorporated in BF_1 were measured after Sephadex G-50 chromatography. [b] [^{14}C]DCCD incorporated in BF_1 was measured on the dissociated enzyme, before DEAE-cellulose chromatography. [c] The number of β subunits per BF_1 was derived from the ratio of the number of moles of [^{14}C]DCCD bound per mole of BF_1 (column 3) to the number of moles of [^{14}C]DCCD bound per mole of β subunit (column 4). [d] The figures reported in the first line correspond to the experiment detailed under Experimental Procedures.

and Degree of Inactivation of BF_1. The second approach to the stoichiometry of the β subunits was by examining the correlation between inactivation and labeling of BF_1 by [^{14}C]DCCD. Here again the interpretation of data relies on the principles used before, i.e., the linear relationship between [^{14}C]DCCD binding and inactivation and full inactivation of BF_1 occurring for 1 mol of [^{14}C]DCCD bound/mol of BF_1. Depending on whether two or three β subunits are present in BF_1, the specific radioactivity of the isolated β subunit is expected to be $^1/_2$ or $^1/_3$ of the specific radioactivity of the whole BF_1.

For the sake of clarity, a typical experiment is described thereafter. BF_1 (7.5 mg) was dialyzed for 3 h at room temperature against 25 mM Mops buffer, pH 6.5. The final volume was adjusted to 10.1 mL with the same buffer. An aliquot of 0.1 mL was withdrawn and supplemented with 1 μL of methanol (control sample); to the remaining 10 mL of solution was added 0.1 mL of 0.5 mM [^{14}C]DCCD in methanol (DCCD sample). The samples were incubated at 30 °C for 1 h. Then, the totality of the control sample and 0.1 mL of the DCCD sample was subjected to a centrifugation–filtration on short columns of Sephadex G-50 equilibrated with 25 mM Mops to separate bound and free [^{14}C]DCCD (cf. Methods). The eluates were assayed for ATPase activity, protein content, and bound [^{14}C]DCCD. In this experiment, the extent of inactivation was 74%, and 0.79 mol of [^{14}C]DCCD was incorporated/mol of BF_1 (cf. line 1, Table II). To the remaining 10 mL of the incubation mixture was added 4.7 g of ammonium sulfate with stirring, and after 30 min at 4 °C, the suspension was centrifuged at 30000*g* for 20 min at 0–4 °C. The clear supernatant was discarded, and the precipitated protein was suspended in 10 mL of a medium made of 50 mM succinate–Tris, 1 M NaCl, 0.25 M $NaNO_3$, 0.1 mM dithiothreitol, and 4 mM EDTA, pH 6.0, as recommended by Dunn & Futai (1980) to dissociate BF_1 into subunits. The entire solution was dialyzed overnight at 4 °C against 0.5 L of the dissociation medium. Protein and radioactivity were assayed on 10- or 20-μL aliquots. At this stage, the protein content was 6.7 mg, and the specific radioactivity corresponded to 0.74 mol of [^{14}C]DCCD bound/mol of BF_1. The BF_1 sample was then quickly frozen in dry ice/acetone and stored overnight at −80 °C. After rapid thawing, it was supplemented with 0.05 mL of 0.2 M ATP, pH 7.4, and 0.4 mL of 0.1 M EDTA, pH 7.4, to give final concentrations of 1 and 8 mM, respectively. The BF_1 solution

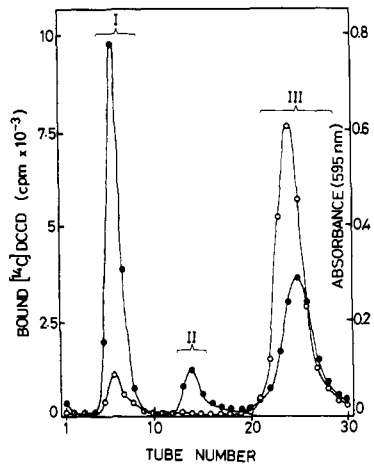

FIGURE 4: Separation of BF_1 subunits by DEAE-cellulose chromatography. BF_1 was inactivated with [^{14}C]DCCD, and the subunits were separated as described under Experimental Procedures. The protein profile (●) and the radioactivity profile (○) are shown.

was then dialyzed at 4 °C against 200 mL of a buffer made of 25 mM sodium succinate, 5 mM ATP, and 2.5 mM 2-mercaptoethanol, pH 6.6, for a period of 2 h with three changes of buffer. Following dialysis, protein and radioactivity were measured. At this stage the total protein content was 5.9 mg, and the specific radioactivity corresponded to 0.76 mol of [^{14}C]DCCD/mol of BF_1 (cf. line 1, Table II). The recovery was quite satisfactory (0.76 mol vs. 0.79 mol in the preceding step). The dialyzed protein solution was chromatographed on a DEAE-cellulose (DE-52 Whatman) column (18 × 1.8 cm), equilibrated with the same buffer as that of the BF_1 solution. The column was eluted by a linear gradient starting with 100 mL of the equilibration buffer, followed by 100 mL of the same buffer supplemented with 0.5 M LiCl. Fractions of 4 mL were collected, and protein and radioactivity were measured on 0.1-mL aliquots. The elution profile is shown in Figure 4. Three fractions were recovered. Fraction I was a mixture of α, γ, and ε subunits. Fraction II was a minor one made of the same subunits. Fractions I and II contained 2.6 mg of protein and 10% of the total bound radioactivity. Fraction III contained only the β subunit; 90% of the bound radioactivity and 2.2 mg of protein were associated with fraction III. The specific radioactivity of fraction III corresponded to 0.25 mol of [^{14}C]DCCD bound/mol of β subunit (line 1 of Table II). The number of β subunits was calculated from the amount of [^{14}C]DCCD bound to F_1 and to the purified β subunit, respectively. Several similar assays of inactivation were performed, differing essentially by the degree of inactivation. The data collected in Table II showed that in seven experiments, the average value found for the number of β subunit per mole of BF_1 was 2.8 ± 0.3 (SEM).

Discussion

As mentioned in the introduction, there is still some uncertainty about the subunit stoichiometry in mitochondrial, bacterial, and chloroplastic F_1. Even the X-ray diffraction measurements, which constitute in principle the most decisive approach, led to some ambiguity (Amzel & Pedersen, 1978; Amzel, 1981). Crystals of rat liver F_1 indicated a molecular two-fold axis of symmetry, which was compatible with a dimeric structure (Amzel & Pedersen, 1978). However, on the basis of a specific arrangement of the different types of subunits, a stoichiometry of the $\alpha_3\beta_3$ type was also considered (Amzel, 1981).

In the specific case of BF_1, the first data on stoichiometry were reported by Bragg & Hou (1975); *E. coli* was grown in

the presence of ^{14}C-labeled amino acids, and the radiolabeled BF_1 was isolated; the distribution of radioactivity on the subunits provided evidence for a stoichiometry of the $\alpha_3\beta_3$ type. Another approach to stoichiometry involved the technique of reconstitution from isolated subunits of *E. coli* BF_1.

Titration of the complex made of the α, γ, and ϵ subunits by isolated β subunits indicated that maximal ATPase activity was recovered for an equimolar amount of the four subunits (Vogel & Steinhart, 1976), suggesting an $\alpha_2\beta_2\gamma_2\epsilon_2$ stoichiometry. However, in other reconstitution experiments, optimal ATPase activity was obtained with the proportion of subunits in accordance in an $\alpha_3\beta_3\gamma$ stoichiometry (Dunn & Futai, 1980). Using isolated F_1 from the thermophilic bacteria PS3, Yoshida et al. (1979) showed that there were three cysteinyl residues in the enzyme and that these residues were located in the α subunits; they concluded that there were three α subunits per F_1.

In the work described here, the number of β subunits in BF_1 was determined by a methodology relying on covalent chemical modification, and in particular, on the partial site reactivity of BF_1 with respect to DCCD. The partial site reactivity of BF_1 was demonstrated for inactivation of BF_1 not only with DCCD (Satre et al., 1979) but also with 4-chloro-7-nitrobenzofurazan (Lunardi et al., 1979). The data were consistent with two or three identical catalytic sites functioning cooperatively at first suggested by Repke et al. (1974) and later demonstrated by Kayalar et al. (1977) and Grubmeyer & Penefsky (1981). A strict correlation was observed between the [^{14}C]DCCD incorporation and the loss of ATPase activity. During the inactivation process, the unstable *O*-acylisourea intermediates are likely to be rearranged to the stable *N*-acylurea derivatives since the covalently bound reagent withstands $NaDodSO_4$–polyacrylamide gel electrophoresis (Satre et al., 1979; Pougeois et al., 1979). Two methods were used to assess the number of β subunits in BF_1. The first one was based on the separation of β and β-DCCD polypeptides by electrofocusing; the second one relied on direct comparison, after inactivation of BF_1 by [^{14}C]DCCD, of the specific radioactivities of the whole BF_1 and the isolated β subunits. The data indicated a stoichiometry of three β subunits per mole of F_1. One could argue that some overestimation could have occurred because of loss in bound DCCD during the isolation of the β subunits. This is, however, not likely since the *N*-acylurea derivatives arising from reaction of DCCD with carboxyl groups are very stable (Williams & Ibrahim, 1981). As bacterial, mitochondrial, and chloroplastic F_1-ATPases exhibit extended biochemical similarities, it is tempting to assume that they should all contain three β subunits per mole of F_1. However, whereas there is strong experimental evidence for a trimeric structure of BF_1, and possibly of mitochondrial F_1, there are a number of data that support the dimeric stoichiometry of chloroplastic F_1 [for a review, see Shavit (1980)]. It is therefore not impossible that during the course of evolution the α and β subunits of F_1 and structurally related proteins (Weltman & Dowben, 1973) have evolved with typically specific quaternary structures resulting in dimers, trimers, and even polymers, as in the case of tubulin in microtubules.

References

Amzel, L. M. (1981) *J. Bioenerg. Biomembr. 13*, 109–121.

Amzel, L. M., & Pedersen, P. L. (1978) *J. Biol. Chem. 253*, 2067–2069.

Binder, A., Jagendorf, A., & Ngo, E. (1978) *J. Biol. Chem. 253*, 3094–3100.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bragg, P. D., & Hou, C. (1975) *Arch. Biochem. Biophys. 167*, 311–321.

Catterall, W. A., Coty, W. A., & Pedersen, P. L. (1973) *J. Biol. Chem. 248*, 7427–7431.

Dunn, S. D., & Futai, M. (1980) *J. Biol. Chem. 255*, 113–118.

Dunn, S. D., & Heppel, L. A. (1981) *Arch. Biochem. Biophys. 210*, 421–436.

Esch, F., & Allison, W. S. (1979) *J. Biol. Chem. 254*, 10740–10746.

Esch, F., Böhlen, P., Otsuka, A. S., Yoshida, M., & Allison, W. S. (1981) *J. Biol. Chem. 256*, 9084–9089.

Futai, M., & Kanazawa, H. (1980) *Curr. Top. Bioenerg. 10*, 181–215.

Gregory, R., & Hess, B. (1981) *FEBS Lett. 129*, 210–214.

Grubmeyer, C., & Penefsky, H. S. (1981) *J. Biol. Chem. 256*, 3728–3734.

Huberman, M., & Salton, M. R. J. (1979) *Biochim. Biophys. Acta 547*, 230–240.

Kagawa, Y., Sone, N., Yoshida, M., Hirata, H., & Okamoto, H. (1976) *J. Biochem. (Tokyo) 80*, 141–151.

Kagawa, Y., Ohta, S., Yoshida, M., & Sone, N. (1980) *Ann. N.Y. Acad. Sci. 358*, 103–117.

Kayalar, C., Rosing, J., & Boyer, P. D. (1977) *J. Biol. Chem. 252*, 2486–2491.

Lunardi, J., Satre, M., Bof, M., & Vignais, P. V. (1979) *Biochemistry 18*, 5310–5316.

Nelson, N. (1976) *Biochim. Biophys. Acta 456*, 314–338.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Patterson, M. S., & Greene, R. C. (1965) *Anal. Chem. 37*, 854–857.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Pougeois, R., Satre, M., & Vignais, P. V. (1979) *Biochemistry 18*, 1408–1413.

Repke, K. R. H., Dittrich, F., & Schön, R. (1974) *Acta Biol. Med. Ger. 33*, 39–47.

Sarraste, M., Gay, N. J., Eberle, A., Runswick, M. J., & Walker, J. E. (1981) *Nucleic Acids Res. 9*, 5287–5296.

Satre, M., Lunardi, J., Pougeois, R., & Vignais, P. V. (1979) *Biochemistry 18*, 3134–3140.

Senior, A. E. (1975) *Biochemistry 14*, 660–664.

Shavit, N. (1980) *Annu. Rev. Biochem. 49*, 111–138.

Stutterheim, E., Henneke, M. A. C., & Berden, J. A. (1981) *Biochim. Biophys. Acta 634*, 271–278.

Takeshige, K., Hess, B., Böhm, M., & Zimmerman-Telschow, H. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1605–1622.

Vogel, G., & Steinhart, R. (1976) *Biochemistry 15*, 208–215.

Wakabayashi, T., Kubota, M., Yoshida, M., & Kagawa, Y. (1977) *J. Mol. Biol. 117*, 515–519.

Weltman, J. K., & Dowben, R. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3230–3234.

Williams, A., & Ibrahim, I. T. (1981) *Chem. Rev. 81*, 589–636.

Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1978) *Biochem. Biophys. Res. Commun. 84*, 117–122.

Yoshida, M., Sone, N., Hirata, H., Kagawa, Y., & Ui, N. (1979) *J. Biol. Chem. 254*, 9525–9533.

Yoshida, M., Poser, J. W., Allison, W. S., & Esch, F. S. (1981) *J. Biol. Chem. 256*, 148–153.