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Aurovertin Binding Sites on Beef Heart Mitochondrial F_1-ATPase. Study with [^{14}C]Aurovertin D of the Binding Stoichiometry and of the Interaction between Aurovertin and the Natural ATPase Inhibitor for Binding to F_1†

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ABSTRACT: Chemically ^{14}C-labeled aurovertin D [Issartel, J. P., Klein, G., Satre, M., & Vignais, P. V. (1983) *Biochemistry* (preceding paper in this issue)] was used to study directly the interaction of aurovertin D with isolated beef heart mitochondrial F_1-ATPase by an equilibrium dialysis technique. With this assay, it was shown that each F_1 possesses three aurovertin sites; one of them had a much higher affinity than the other two (K_d = 0.2–0.3 μM vs. 3–5 μM). Isolated β subunit was found to bind [^{14}C]aurovertin D with a K_d of about 1 μM and a stoichiometry of 1 mol of aurovertin/mol of β subunit. As each F_1 binds three molecules of [^{14}C]aurovertin D, it follows that F_1 contains three β subunits. The aurovertin binding capacity of F_1 was not modified by addition of ADP or ATP. On the other hand, the binding affinity of F_1 for [^{14}C]aurovertin D was more affected by ATP than by ADP. In the presence of ATP, two relatively high-affinity sites ($K_d \simeq 0.7$ μM) and one low-affinity site ($K_d \simeq 5$ μM) were titrated. The binding data obtained by the isotopic technique were compared with those calculated from the classical fluorescence assay. The latter assay allowed the detection of only one high-affinity site ($K_d \simeq 0.2$ μM) and one low-affinity site ($K_d \simeq 5$ μM); upon addition of ATP, two sites of high affinity ($K_d \simeq 0.3$ μM) but none of low affinity were detected. The effect of the natural ATPase inhibitor, IF_1, on the binding of [^{14}C]aurovertin D was followed under conditions that allowed nearly full inhibition of the ATPase activity of F_1 by IF_1; this corresponds to the binding of 1 molecule of IF_1 to 1 molecule of F_1 at the level of the β subunit [Klein, G., Satre, M., Dianoux, A.-C., & Vignais, P. V. (1980) *Biochemistry 19*, 2919–2925]. Under these conditions the binding affinity of all three sites of F_1 for [^{14}C]aurovertin D was strongly decreased.

In the preceding paper (Issartel et al., 1983), a procedure for the chemical radiolabeling of aurovertin D was described; appropriate binding assays with *Escherichia coli* BF_1[1] and an aurovertin-insensitive mutant were reported, demonstrating the specific character of [^{14}C]aurovertin D binding to BF_1. The aim of the present work was to apply the same isotopic assay to the study of the interaction of aurovertin D with mitochondrial F_1. So far, all attempts to analyze the binding features of aurovertin D to mitochondrial F_1 have been based on the enhancement of the fluorescence of the aurovertin molecule upon binding to F_1 (Lardy & Lin, 1969). The number of moles of bound aurovertin per mole of F_1 was found to be 1 or 2 depending on experimental conditions (Lardy & Lin, 1969; Lardy & Lambeth, 1972; Bertina et al., 1973; Chang & Penefsky, 1973; Müller et al., 1977; Berden & Verschoor, 1978; Stutterheim et al., 1980). It was later demonstrated on the basis of the fluorescence test that aurovertin D binds in a 1 to 1 stoichiometry to the β subunit in beef heart F_1 (Verschoor et al., 1977) and yeast F_1 (Douglas et al., 1977; Stutterheim et al., 1980). As there was one binding site per β subunit and a maximal number of 2 mol of aurovertin bound per mol of F_1 from beef heart or yeast, Verschoor et al. (1977) and Stutterheim et al. (1980) concluded that each F_1 contained two β subunits.

The direct titration of the aurovertin site of F_1 with [^{14}C]aurovertin D described here shows unambiguously that the beef heart isolated β subunit binds 1 aurovertin molecule and that each beef heart F_1 contains three aurovertin binding sites of different affinities, leading to the conclusion that beef heart F_1 contains three β subunits. As an illustration of the use of [^{14}C]aurovertin D, we present a study of the effect of the natural ATPase inhibitor (Pullman & Monroy, 1963) on aurovertin binding to beef heart F_1.

Experimental Procedures

Biological Preparations. Beef heart mitochondria were isolated by the method of Smith (1967). Beef heart mitochondrial F_1 was prepared and stored as an ammonium sulfate precipitate as described by Knowles & Penefsky (1972). Before use, the suspension was centrifuged; the pellet was solubilized in the equilibrium dialysis buffer (see below) and desalted by the centrifugation–filtration method of Penefsky (1977). Molar concentrations of purified F_1 were calculated on the basis of an M_r of 360 000 (Lambeth et al., 1971). The natural ATPase inhibitor (IF_1) was prepared by the method of Horstman & Racker (1970).

Preparation of Isolated β Subunit. The procedure of Dunn & Futai (1980) described for the dissociation of *E. coli* ATPase was applied to the mitochondrial F_1-ATPase. All op-

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[1] Abbreviations: F_1, beef heart soluble mitochondrial ATPase; BF_1, soluble F_1-ATPase isolated from *E. coli*; IF_1, beef heart ATPase protein inhibitor; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

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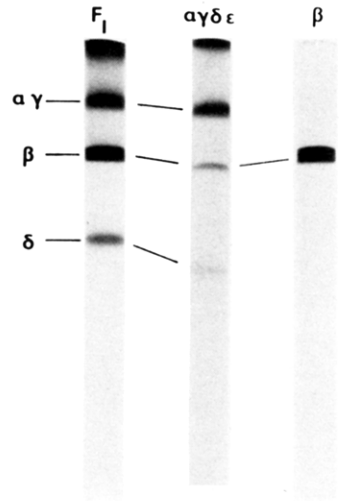

FIGURE 1: Gel electrophoresis of isolated subunits of mitochondrial F_1. 5% polyacrylamide gel electrophoresis in the presence of 8 M urea was performed as described by Knowles & Penefsky (1972). Lane 1 corresponds to control F_1, lane 2 to $\alpha\gamma\delta\epsilon$ complex, and lane 3 to the β subunit. Note the slight contamination of the $\alpha\gamma\delta\epsilon$ complex by β subunit.

erations were carried out at 2–4 °C. A sample of F_1 (15 mg) in ammonium sulfate (see above) was centrifuged for 2 min in an Eppendorf centrifuge. The pellet was solubilized in 10 mL of a dissociation medium containing 50 mM succinate–Tris, 1 M NaCl, 0.25 M $NaNO_3$, 0.1 mM dithiothreitol, and 4 mM EDTA, final pH 6.1, and dialyzed overnight against 1 L of the same medium. The dialyzed fraction was then frozen and stored at −80 °C for 24 h. To fractionate the subunits from the dissociated F_1, the method described by Vogel & Steinhart (1976) for *E. coli* ATPase was applied. The frozen sample of F_1 was thawed and dialyzed for 2 h against 1.2 L of a buffer containing 25 mM succinate, 4 mM EDTA, 5 mM ATP, and 2.5 mM 2-mercaptoethanol, final pH 6.5 (buffer A). The dissociated F_1 was then layered on top of a column of DEAE-cellulose (DE-52, Whatman) equilibrated with buffer A and eluted with a continuous LiCl gradient (0–0.5 M) in buffer A. Two major peaks were recovered, the first one corresponding to an $\alpha\gamma\delta\epsilon$ complex and the second to pure β subunit (Figure 1).

Isolation of Aurovertin D and Synthesis of [^{14}C]Aurovertin D. Aurovertin D was purified from cultures of *Calcarisporium arbuscula* (NRRL 3705) as described by Osselton et al. (1974). The chemical radiolabeling of aurovertin D was performed by replacement of the acetyl residue of the molecule by a [^{14}C]acetyl group. The concentrations of the aurovertin solutions were determined by using the extinction coefficient of 36 100 M^{-1} cm^{-1} at 368 nm, calculated from the specific radioactivity of [^{14}C]aurovertin D (Issartel et al., 1983).

Aurovertin Binding Assays. Binding of aurovertin D was measured either by a direct isotopic assay based on equilibrium dialysis of [^{14}C]aurovertin D in the presence of F_1 at 20 °C, as described in the preceding paper (Issartel et al., 1983), or by a fluorescence assay based on the variation of the aurovertin fluorescence upon binding to F_1. In the isotopic assay, the following standard medium was used: 0.25 M sucrose, 10 mM Tris–sulfate, 0.5 mM EDTA, and 10% (v/v) methanol, pH 7.4. Additions of nucleotides are specified in the legends. Methanol was added to a final concentration of 10% (v/v) to stabilize the enzyme (Schuster, 1979) during the 90-min period required for equilibration in the dialysis assay. Aliquot fractions (from 0.03 to 0.08 mL) were withdrawn from the dialysis cell and mixed with 1 mL of 50% methanol and 10 mL of a toluene–Triton X-100 scintillation fluid (Patterson & Greene, 1965). Radioactivity was counted with an Intertechnique SL 30 scintillation counter.

In fluorescence assays, the binding parameters of aurovertin D to F_1 were measured by monitoring the changes of aurovertin fluorescence upon binding, applying the rationale of Müller et al. (1977). Fluorescence measurements were made at 20 °C with a Perkin-Elmer MPF-2A fluorometer; the excitation wavelength was set at 365 nm and the emission wavelength at 470 nm. As explained in the preceding paper (Issartel et al., 1983), the amount of bound aurovertin, a_b, was calculated by the expression $(F_{obsd} - \alpha_f a)/\alpha_b$, where α_f and α_b are the fluorescence coefficients [arbitrary units (μM^{-1})] for free and bound aurovertin, a is the concentration of total aurovertin, and F_{obsd} is the fluorescence intensity observed after mixing aurovertin and F_1. The term $\alpha_f a$ was the measured fluorescence in the absence of F_1; α_b [arbitrary units (μM^{-1})] was obtained by plotting the reciprocal of the fluorescence intensity vs. the reciprocal of the F_1 concentration for a given concentration in aurovertin and extrapolating to infinite concentration of F_1.

ATPase Assay. F_1-ATPase activity was measured at 30 °C in 0.5 mL of a medium containing 50 mM Tris–SO_4, 10 mM ATP, 5 mM $MgCl_2$, 70 μg/mL pyruvate kinase, and 10 mM phosphoenolpyruvate (tricyclohexylammonium salt), final pH 8.0. The reaction was stopped by addition of 0.1 mL of 2.5 N perchloric acid. The released P_i was determined by the method of Fiske & SubbaRow (1925). The specific activities of the F_1 preparations used in this work ranged between 85 and 95 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$.

Protein Determination. F_1-ATPase concentrations were assayed with the Coomassie G250 binding method (Bradford, 1976), using bovine serum albumin as a standard. It has been shown (Kasahara & Penefsky, 1978) that protein concentration determined by the Coomassie Blue method agreed within 5% with the dry weight of F_1.

Results

Binding of [^{14}C]Aurovertin D to Isolated F_1 As Measured by Equilibrium Dialysis. In the standard medium without further addition, titration of F_1 with [^{14}C]aurovertin D showed a maximal binding of 3 mol of aurovertin/mol of F_1 (Figure 2A). The corresponding Scatchard plot (Figure 2B) was nonlinear, consistent with the presence of high- and low-affinity sites. Graphical analysis of the plot by the method of Rosenthal (1967) yielded the following parameters: one high-affinity site with a K_d of 0.2 μM and two low-affinity sites with K_d values of 4–5 μM. Addition of ADP or ATP did not modify the overall binding stoichiometry of aurovertin D to F_1 (Figure 2C,D). On the other hand, whereas ADP had no significant effect on the site affinity, ATP increased the affinity of one of the two low-affinity sites and decreased that of the high-affinity site (Figure 2C); thus F_1 incubated with ATP exhibited two relatively high affinity sites (K_d = 0.7 μM) and one low-affinity site (K_d = 5 μM). When Mg^{2+} was present together with ATP, the two high-affinity sites were still expressed and the K_d value was 0.2 μM; the low-affinity site was also present. The latter conditions, i.e., $MgCl_2$ plus ATP, were those utilized for binding of the ATPase inhibitor to F_1, as described hereafter. The aurovertin binding parameters under the different conditions described above are listed in Table I.

Binding of Aurovertin D to F_1 As Measured by Fluorescence Changes. The fluorescence technique was compared to the isotopic technique with respect to determination of the aurovertin binding parameters. For this purpose, similar incubation conditions were used; i.e., F_1 was preincubated for

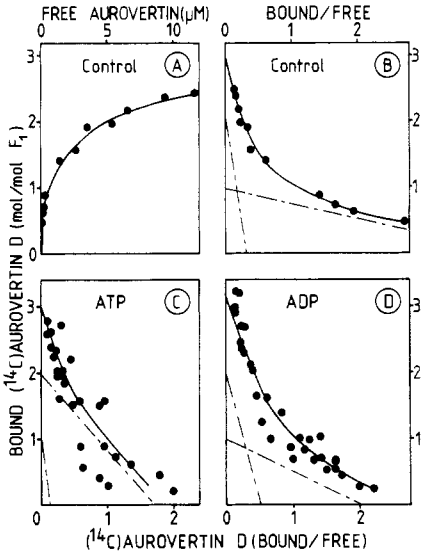

FIGURE 2: Binding of [^{14}C]aurovertin D to F_1 as measured by equilibrium dialysis. (A) F_1 (180 μL, 0.72 μM) was dialyzed for 90 min against 180 μL of standard medium containing increasing concentrations of [^{14}C]aurovertin D in the absence of nucleotides. Binding of [^{14}C]aurovertin D was determined as described under Experimental Procedures. (B) Scatchard plot of the binding data. The curve drawn through experimental data points is the simulated curve calculated from partial plots (dotted lines) of three independent classes of binding sites of different affinities, one class of high affinity and two classes of low affinity. To simplify the decomposition of the binding curve and the calculation of the binding parameters, the two classes of low-affinity sites were brought together, so that only two classes of sites, high and low affinity were considered in calculation. The amount of [^{14}C]aurovertin D bound to F_1 with high affinity (B_1) and low affinity (B_2) was related to the total number of high-affinity sites (N_1) and low-affinity sites (N_2), with respective affinities K_1 and K_2 and to the concentration of unbound [^{14}C]aurovertin, U, by the relation of $B_1 + B_2 = N_1U/(K_1 + U) + N_2U/(K_2 + U)$. The Scatchard curve was drawn with the following parameters: $N_1 = 1$, $K_1 = 0.18$ μM, $N_2 = 2$, and $K_2 = 4.5$ μM. As indicated above, it was assumed that the two low-affinity sites have the same K_d values. (C) Scatchard plot of [^{14}C]aurovertin D binding to F_1 in the presence of 2 mM ATP; $F_1 = 0.61$ μM, $N_1 = 2$, $K_1 = 0.7$ μM, $N_2 = 1$, and $K_2 = 5.1$ μM. (D) Scatchard plot of [^{14}C]aurovertin D binding to F_1 in the presence of 0.2 mM ADP: $F_1 = 0.69$ μM, $N_1 = 1$, $K_1 = 0.33$ μM, $N_2 = 2$, and $K_2 = 2.7$ μM.

Table I: Binding Parameters of [^{14}C]Aurovertin D to F_1 [a]

additions	no.[c] of high-affinity sites and K_d values (μM)[d]	no.[c] of low-affinity sites and K_d values (μM)[d]
none	1 (0.15–0.5)	2 (4–5)
ADP[b]	1 (0.25–0.35)	2 (2–3)
ATP[b]	2 (0.7–1)	1 (4–6)
ATP + $MgCl_2$[b]	2 (0.2–0.3)	1 (2–3)

[a] The isotopic assay was performed as described under Experimental Procedures. At least two titrations were carried out for each condition. [b] ADP was added at a final concentration of 0.2 mM. When added alone, ATP was 2 mM. When added in the presence of $MgCl_2$, ATP was 0.5 mM and $MgCl_2$ 1 mM. The pH was 7.4, except for the latter condition (ATP + $MgCl_2$), where it was 6.5. [c] The integral numbers of sites of high and low affinity were used as a basis for the mathematical analysis of the [^{14}C]-aurovertin binding curve. [d] The K_d values are given in parentheses.

90 min in the standard medium, a step mimicking the 90-min period of equilibrium dialysis with [^{14}C]aurovertin D, prior to the addition of aurovertin and measurement of fluorescence. The direct binding curves in the absence of nucleotides and the corresponding Scatchard plot are shown in Figure 3A,B. The extrapolated Scatchard plot indicated two aurovertin

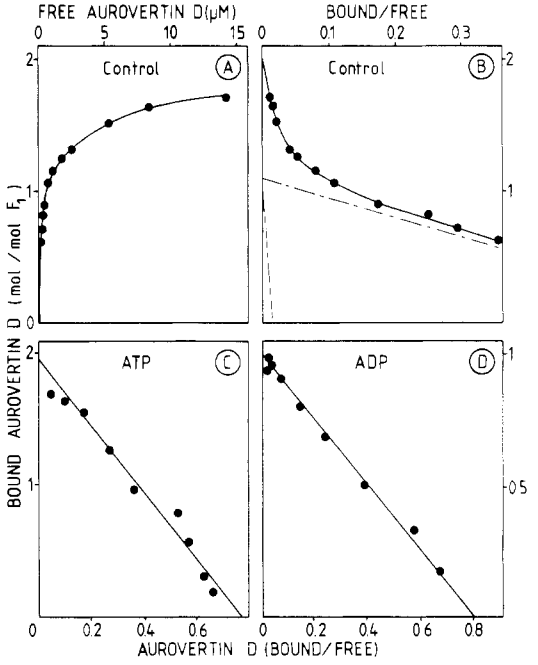

FIGURE 3: Binding of aurovertin D to F_1, as measured by fluorescence. F_1 (0.72 μM) was incubated for 90 min in the standard medium in the absence of nucleotide. The aurovertin fluorescence was measured in a cuvette containing 2 mL of standard medium. To determine α_b (see Experimental Procedures), 0.13 μM aurovertin D was incubated with increasing concentrations of F_1 (up to 0.13 μM); α_b was obtained from the reciprocal plot of the fluorescence intensity (F) vs. the reciprocal of F_1 concentration. F_1 (70 nM) was then titrated with increasing concentrations of aurovertin D (up to 8 μM). (A) Direct titration data. (B) Scatchard plot of the binding data. The Scatchard plot was resolved into two classes of binding sites as described in the legend of Figure 2. The curve drawn through the experimental points was obtained by using two classes of binding sites as shown by the dotted lines corresponding to the following parameters: $N_1 = 1.1$, $K_1 = 0.1$ μM, $N_2 = 1.0$, and $K_2 = 5$ μM. (C) Scatchard plot of the binding data in the presence of 2 mM ATP: $F_1 = 151$ nM, $N = 2$, and $K = 0.4$ μM. (D) Scatchard plot of the binding data in the presence of 0.2 mM ADP: $F_1 = 94$ nM, $N_1 = 1$, and $K_1 = 0.1$ μM. Note the different scales of abscissa for panels B–D.

binding sites. Analysis by the graphical method of Rosenthal (1967) yielded two straight lines, reflecting the titration of one high-affinity site ($K_d = 0.1$ μM) and one low-affinity site ($K_d = 5$ μM), respectively. In the presence of 0.2 mM ADP (Figure 3D), the high-affinity site remained unaltered, and the low-affinity site vanished. On the other hand, in the presence of 2 mM ATP, the binding data were strikingly modified; two binding sites of equal affinities ($K_d \simeq 0.3$–0.4 μM) were revealed (Figure 3C), instead of the two distinct high- and low-affinity sites on the control enzyme.

In complementary fluorescence assays carried out without the 90-min preincubation step, the high-affinity aurovertin site was still titrated; however, the low-affinity site was not revealed unless ATP was added. In fact, addition of ATP with or without Mg^{2+} resulted in the unmasking of a second high-affinity site. The K_d values of the two high-affinity sites titrated under these conditions were very close to each other and virtually indistinguishable, 0.5 μM in the case of ATP added alone and 0.1 μM in that of ATP added in the presence of $MgCl_2$.

Comparison of data obtained by the isotopic and fluorescence techniques makes it clear that the isotopic test was able to detect low-affinity sites that were hardly detectable or not detectable at all by fluorometry. One remarkable result in both isotopic and fluorometric assays was the modulation of the aurovertin binding parameters in the presence of ATP,

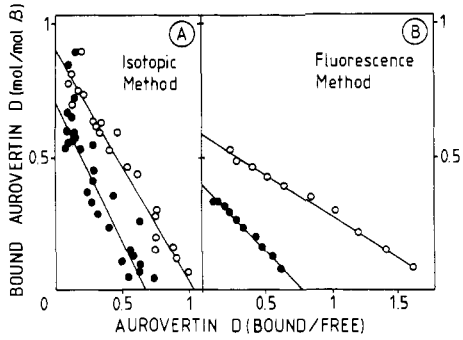

FIGURE 4: Binding of aurovertin D to isolated β subunit. The binding of aurovertin D to isolated β subunit was measured in the standard medium without methanol either by equilibrium dialysis of [^{14}C]-aurovertin D (panel A) or by fluorescence (panel B) in the absence (●) or in the presence of 2 mM ADP (○). The β subunit was used at a concentration of 1.4 μM in the isotopic assay and 0.7 μM in the fluorescence assay.

which consisted essentially in a doubling of the number of the high-affinity sites accompanied by an increase in the K_d value.

Aurovertin D Binding to Isolated β Subunit. As shown in Figure 4A, [^{14}C]aurovertin D was able to bind to the isolated β subunit with a stoichiometry of 0.7 mol/mol of β subunit and a K_d value of 1.1–1.3 μM. Addition of ADP (2 mM) increased the stoichiometric ratio to 0.9, the K_d remaining the same. It is noteworthy that in the case of the isolated β subunit, ADP had to be added at higher concentration than in the case of F_1 to elicite a response in the binding properties of aurovertin D. With the fluorescence technique (Figure 4B), the amount of bound aurovertin D was significantly less than that found by the isotopic technique, namely, 0.4 and 0.6 mol of β subunit in the absence and presence of ADP; the K_d values were 0.4 μM and 0.2 μM, respectively. The low stoichiometry found in the fluorescence assay may be due to inaccuracy in the determination of the fluorescence intensity by extrapolation to infinite protein concentration, especially when the plots are nonlinear.

Effect of Natural ATPase Inhibitor (IF_1) on Binding of Aurovertin D to F_1. It is known that the natural ATPase inhibitor binds to the β subunit of F_1 and that full inhibition of F_1 by IF_1 requires the binding of 1 mol of IF_1/mol of F_1 in spite of the fact that F_1 contains three β subunits (Klein et al., 1980, 1981b). The binding of [^{14}C]aurovertin D to the isolated F_1 and the F_1–IF_1 complex was investigated under the optimal conditions for formation of the F_1–IF_1 complex, i.e., a slightly acidic medium supplemented with Mg–ATP (Horstman & Racker, 1970).

As shown previously (Table I), in the presence of Mg–ATP, each mol of F_1 was able to bind 3 mol of [^{14}C]aurovertin D. Two of them were bound with high affinity (K_d = 0.2–0.3 μM), and the remaining one was bound with a lower affinity (K_d = 2–3 μM). In the experiment described in Figure 5, the amount of IF_1 added to F_1 was such that 93% of the ATPase activity of F_1 was inhibited, which corresponds to the binding of 0.93 mol of IF_1 to 1 mol of F_1 (Klein et al., 1980); the maximal binding capacity was still apparently of 3 aurovertin molecules per F_1 in the F_1–IF_1 complex, but the binding affinity was considerably decreased (K_d = 2–3 μM).

The loss of aurovertin affinity of the IF_1–F_1 complex shown by the isotopic assay was corroborated by a fluorescence experiment, as illustrated in Figure 6. In this experiment, F_1 was preincubated with increasing concentrations of IF_1, and aurovertin D was added further at a fixed concentration. To better determine the interfering effect of IF_1 on the affinity of F_1 for aurovertin D, this ligand was used at a relatively low

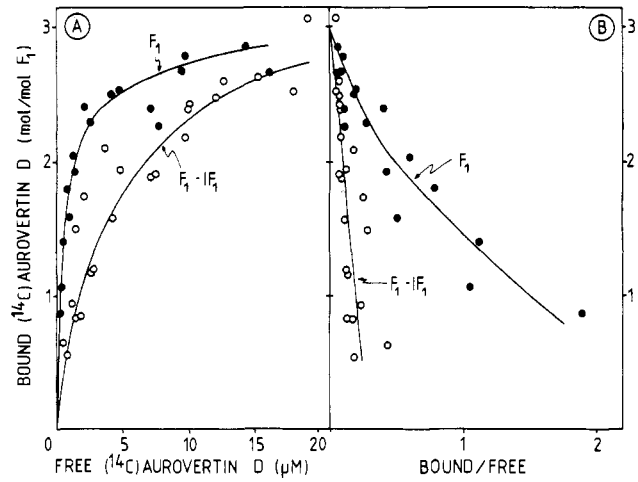

FIGURE 5: Effect of natural protein inhibitor IF_1 on [^{14}C]aurovertin D binding to F_1. F_1 (0.7 μM) was preincubated for 15 min at 25 °C in the absence or in the presence of IF_1 (2 μM) in a medium containing 0.25 M sucrose, 10 mM Mops, 1 mM $MgCl_2$, and 0.5 mM ATP, final pH 6.5. The ATPase activity of control F_1 and the degree of inhibition of the F_1–IF_1 complex were determined on aliquot fractions (the ATPase inhibition caused by IF_1 was 93%). The remaining was used for determination of bound [^{14}C]aurovertin D by the equilibrium dialysis technique; in this assay, F_1, either alone or in the F_1–IF_1 complex, was used at the final concentration of 0.3 μM. The dialysis assay was performed in a medium consisting of 0.25 M sucrose, 10 mM Mops, pH 6.5, and 10% (v/v) methanol. It was checked that the ATPase activity of control F_1 was constant over the period of 90 min and that the inhibition of the ATPase activity induced by IF_1 in the F_1–IF_1 complex was not reversed after the dialysis period. (A) (●) Binding of [^{14}C]aurovertin D to control F_1; (○) binding of [^{14}C]aurovertin D to F_1–IF_1 complex. (B) Scatchard plot of the binding data: (●) control F_1; (○) F_1–IF_1 complex. The theoretical curve passing through the experimental points (●) was calculated by assuming two classes of binding sites corresponding to the following parameters: for F_1, $N = N_1 + N_2 = 3$, $N_1 = 2$, $K_1 = 0.23$ μM, $N_2 = 1$, and $K_2 = 2.3$ μM; in the case of the F_1–IF_1 complex $N = 3$ and $K_d = 2.5$ μM.

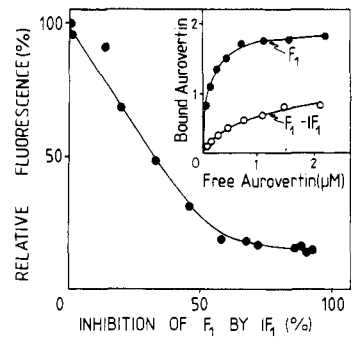

FIGURE 6: Correlation between loss of aurovertin fluorescence and fractional inhibition of F_1 by IF_1. 1.2 μM F_1 was incubated for 15 min in 100 μL of the IF_1 binding medium containing 0.25 M sucrose, 10 mM Mops, pH 6.5, 1 mM $MgCl_2$, and 0.5 mM ATP with increasing amounts of IF_1. The ATPase activity was measured on aliquots (2 μL), and the aurovertin fluorescence was assayed in 0.25 M sucrose/10 mM Mops, pH 6.5, on the remaining fraction at an aurovertin D concentration of 0.36 μM as described under Experimental Procedures. It was checked that the fluorescence of bound aurovertin at infinite protein concentration was unchanged for F_1 or the F_1–IF_1 complex. In the inset of the figure are shown the direct binding curves of aurovertin D to F_1 or the F_1–IF_1 complex. F_1 was preincubated for 15 min in the IF_1 binding medium without IF_1 (●) or with IF_1 (○). ATPase inhibition of F_1 by IF_1 was 83%.

concentration, 0.36 μM. The data show that the amount of bound aurovertin decreased linearly as a function of the inhibition of F_1 by IF_1 to practically 70–80% inhibition. A complementary experiment of titration of F_1 in the IF_1–F_1 complex by increasing concentrations of aurovertin was per-

formed, F_1 being inhibited to about 80% by IF_1 (Figure 6, inset); clearly, not only the binding capacity but also the affinity for aurovertin were altered; one site disappeared, and the K_d of the remaining site was markedly increased. In brief, the results of the fluorescence and isotopic assays led to the conclusion that binding of IF_1 to F_1 decreases aurovertin binding to F_1. However, they differed in that one site in the IF_1–F_1 complex was no longer detectable by the fluorescence assay but was still revealed by the isotopic assay. As mentioned above, the ambiguity of the fluorescence assay rests on the difficulty of evaluation of the aurovertin low binding affinity, which was not the case for the isotopic technique.

Discussion

An analysis of the data for binding of [^{14}C]aurovertin D to beef heart F_1 indicates unambiguously that each F_1 possesses three aurovertin sites. One of the three sites on F_1 is a high-affinity site with a K_d of about 0.2 μM, the K_d of the other two sites being in the range of 4–5 μM. Addition of ADP had no significant effect on the binding of aurovertin D to F_1; in contrast, addition of ATP resulted in the transformation of a low-affinity aurovertin site into a high-affinity site (K_d = 0.3 μM). There are some gross similarities between the binding features of the aurovertin sites in mitochondrial F_1 and in *E. coli* BF_1 (Issartel et al., 1983); notably, a maximal number of three aurovertin sites were found in both cases, one of the three sites having a higher affinity for [^{14}C]aurovertin D than the other two.

The binding experiments with [^{14}C]aurovertin D demonstrated clearly the existence of low-affinity sites that escaped detection by fluorescence assay. In fact, with the fluorescence technique, the aurovertin D binding stoichiometry (moles of bound aurovertin/mole of F_1) was found to be 1 in the absence of ATP or 2 in the presence of ATP, in the absence of any preincubation, in agreement with Chang & Penefsky (1973). Unexpectedly, after a 90-min preincubation period in the presence of 10% methanol, the binding stoichiometry found by fluorescence increased to 2 molecules of aurovertin D bound per F_1 in the absence of ATP; however, the second molecule of aurovertin bound with low affinity. It is noteworthy that, with yeast mitochondrial F_1, Stutterheim et al. (1980) observed, in contrast to beef heart F_1, a decrease in the concentration of detectable aurovertin binding sites upon incubation with methanol. There is no obvious explanation for the different effect of methanol on aurovertin binding to yeast and beef heart F_1. The rise from 1 to 2 aurovertin sites on beef heart F_1 by preincubation with methanol can be compared to that found by Müller et al. (1977), who observed two aurovertin sites per F_1 in the absence of ATP, using an enzyme precipitated 4 times with ammonium sulfate, a treatment that is expected to deplete F_1 in bound nucleotides (Harris et al., 1973). Likewise, a possible release of bound nucleotides during the preincubation step of F_1 in the standard medium or a slight modification in the arrangement of the F_1 subunits, especially in that of the β subunits during preincubation, may result in elicitation of a stronger interaction of aurovertin D with a second β subunit and, consequently, in a fluorescence increase.

A stoichiometry of 0.7–0.9 mol of [^{14}C]aurovertin D/mol of β subunit (K_d = 1.2–1.3 μM) was found by the direct isotopic assay, suggesting the presence of 1 aurovertin binding site per β subunit. This result and the fact that aurovertin binds only to the β subunit in mitochondrial F_1 (Douglas et al., 1977; Verschoor et al., 1977) strongly suggests that there are three β subunits per F_1.

As in the case of the bacterial BF_1, one may ask whether the heterogeneity of the three aurovertin D sites on the mitochondrial F_1 reflects the existence of independent heterogeneous sites or that of interacting homogeneous sites. Titration of the aurovertin site of isolated β subunits by the isotopic technique failed to detect significant heterogeneity. This suggests therefore an interaction between homogeneous aurovertin sites, the binding of 1 molecule of aurovertin D to one β subunit resulting in a decreased affinity of the other subunits for aurovertin D. However, as discussed by Amzel et al. (1982), a specific contact between one of the β subunits in F_1 and one of the minor subunits (for example, γ) may result in nonequivalence of this β subunit with respect to the other two. This would explain the existence per F_1 of one aurovertin high-affinity site in spite of three identical β subunits. In brief, the differences in aurovertin binding affinity for the three β subunits of F_1 may be explained either by a mechanism of true anticooperativity or by a contact-induced change of conformation that makes one of the β subunits in F_1 nonequivalent to the other two.

The binding of a number of ligands to the aurovertin–F_1 complex has been shown to elicit in many cases a change in the fluorescence intensity of bound aurovertin D. Conversely, the binding of aurovertin D to F_1 may result in modification of the binding affinity and capacity for other ligands, which most likely reflects conformational changes of F_1. Along this line, a number of data have been reported on interaction between the natural ATPase inhibitor and aurovertin D for binding to F_1. For example, when sonic particles rich in IF_1 are treated with aurovertin D, the lag phase that precedes the onset of oxidation phosphorylation is shortened (Harris et al., 1979); this was explained by an aurovertin-induced release of IF_1. This interpretation was in line with previous observations by Van de Stadt & Van Dam (1974) showing that aurovertin D facilitated the dissociation of the ATPase inhibitor induced by the functioning of the respiratory chain and that the fluorescence of aurovertin bound to IF_1-rich sonic particles was much less than that of aurovertin bound to IF_1-depleted sonic particles. On the other hand, on the basis of a fluorescence assay, Chang & Penefsky (1974) concluded that upon binding of IF_1 to F_1, the K_d value for aurovertin D was decreased from 100 nM to 46 nM and the number of moles of bound aurovertin D per mole of F_1 from 1.8 to 0.84. This is corroborated by the quenching of fluorescence of F_1-bound aurovertin, which is induced by binding of IF_1 (Lowe & Beechey, 1981). Our binding measurements at equilibrium by the isotopic assay show that the three aurovertin sites of F_1 titrated by [^{14}C]aurovertin D are affected in their K_d values by the binding of only 1 molecule of IF_1 per F_1, which, by itself, was sufficient to fully inhibit the F_1-ATPase activity (Klein et al., 1980). These data taken together are consistent with an IF_1-induced conformational change in the whole F_1 molecule, as previously suggested by modification of the pattern of cross-linking between F_1 subunits in the presence of IF_1 and by loss of exchangeability of the loosely bound nucleotides and phosphate in the F_1–IF_1 complex (Klein et al., 1980, 1981a).

As discussed above, the uncertainties concerning the binding stoichiometry of aurovertin D to F_1 examined by fluorometry were most likely due to the inability of the fluorescence technique to detect low-affinity site(s) of F_1. Although the fluorescence method of determination of bound aurovertin has some limitations with respect to the exact determination of the aurovertin binding parameters, it remains a choice method to follow fast conformational changes of isolated or bound F_1, due to the ability of aurovertin to behave as a rapidly responding and highly specific fluorescent probe of F_1.

Registry No. ATPase, 9000-83-3; aurovertin D, 65256-31-7.

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Thermotropic Behavior of Aqueous Dispersions of Glucosylceramide–Dipalmitoylphosphatidylcholine Mixtures†

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ABSTRACT: Differential scanning calorimetry has been carried out on aqueous dispersions of multilamellar liposomes formed from mixtures of glucosylceramide and dipalmitoylphosphatidylcholine. The phase diagram constructed from data obtained from dispersions in excess water in the cooling mode is simpler than the diagram obtained from data obtained in the heating mode. The essentially horizontal portion of the solidus curve up to 60% glucosylceramide indicates lateral phase separation in the gel phase. The existence of a metastable region of glucosylceramide seen in the heating scans at system concentrations greater than 90% of this component is reminiscent of the complex thermal behavior of pure glucosylceramide dispersions in excess water.

Gluco- and galactocerebrosides form lamellar arrays in aqueous solutions and exhibit a complex thermotropic behavior (Bunow, 1979; Bunow & Levin, 1980; Freire et al., 1980; Ruocco et al., 1981; Curatolo, 1982). It is generally believed that this behavior, diagramed in Figure 1 (Freire et al., 1980), is due primarily to head group–head group interactions and a hydration–dehydration process involving the head groups (Freire et al., 1980; Ruocco et al., 1981; Skarjune & Oldfield, 1982). The hydrogen-bond network, demonstrated by NMR[1] studies (Skarjune & Oldfield, 1979, 1982), is altered in the

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[1] Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; GlcCer, D-*erythro*-glucocerebroside; TLC, thin-layer chromatography; T_m, temperature at which maximum heat flow is observed; NMR, nuclear magnetic resonance.

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