observe that neither vinylglycine nor OSHS gives any inactivation, which further directs attention to structure VII as the likely inactivating species, rather than other potential conjugated electrophiles common to those two amino acid substrates.

It is also of interest to note that the rate constant for partial inactivation by chloroaminobutyrate (5.8×10^{-4} s^{-1}) is larger than that for inactivation by fluoroaminobutyrate (2.8×10^{-4} s^{-1}). Insofar as these values express a difference in the rate for halide elimination in a rate-determining step, they confirm a mechanism of inactivation by nucleophilic displacement wherein chlorine is expected to be a better leaving group than fluorine. By contrast, α-carbanion-assisted elimination of the halide, to give eventual formation of α-ketobutyrate, appears to favor fluoride over chloride as a leaving group (Table I). These rate differences, both for inactivation and for processing of the haloamino acids to product ketobutyrate, point to the importance of the stabilized α-halo-β-carbanion VII as a focal kinetic intermediate in both catalysis and inactivation.

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Affinity Labeling of Catalytic and Regulatory Sites of Pig Heart Mitochondrial F_1-ATPase by 5′-*p*-Fluorosulfonylbenzoyladenosine†

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ABSTRACT: Pig heart mitochondrial F_1-ATPase is inactivated on incubation with the adenine nucleotide analogue 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA). The inhibition is reversible if the incubation time does not exceed 1 min and depends on the ATP concentration. The Hill number (h = 1.8 ± 0.2) indicates cooperative binding of FSBA. After 1 min of incubation, FSBA produces irreversible inactivation characterized by biphasic kinetics. The initial, rapid phase diminishes the maximal velocity of the enzyme without modifying the K_m for ATP. The pseudo-first-order rate constant of the rapid phase is independent of FSBA concentration between 0.09 and 0.57 mM, indicating a very high affinity for the analogue. In contrast, the pseudo-first-order rate constant for the second, slower phase depends on FSBA concentration with saturating kinetics; this indicates that a reversible enzyme–inhibitor complex is formed before the irreversible reaction, with a K_D (0.23 mM) similar to the K_m for ATP (0.26 mM). The relationship between the incorporation of [*benzoyl*-^{14}C]FSBA during inactivation and the residual activity of the enzyme is also biphasic and gives an extrapolated value of 6 mol bound per mol of enzyme for complete inhibition. A value of 1–4 mol of FSBA bound per mol of enzyme is calculated for the rapid phase and a maximal number of 2 mol bound for the slow phase. The rapid phase of inactivation appears to reflect the binding to regulatory sites, whereas the slow phase appears to correspond to the binding to catalytic sites.

The purpose of this work was to label irreversibly the regulatory and catalytic sites of F_1-ATPase with an analogue of ATP and ADP. The use of such an analogue with an unmodified adenosine moiety is essential for the understanding of catalytic and regulatory mechanisms of the enzyme. It is generally recognized that F_1-ATPase plays a central role in ATP synthesis when integrated in the mitochondrial membrane, this process being very specific for the adenosine moiety. The presence of regulatory sites, able to bind ADP or ATP, has been demonstrated both by the detection of cooperativity in kinetic studies (Godinot et al., 1975; Ebel & Lardy, 1975;

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Schuster et al., 1975; Recktenwald & Hess, 1977) and by measuring the reversible binding of ADP (Hilborn & Hammes, 1973; Pedersen, 1975) and analogues of ADP or ATP (Penefsky, 1974). Schuster et al. (1975) and Pedersen (1975) found two types of nucleotide binding site, the regulatory site being very specific for the adenosine nucleotide analogues whereas the catalytic site was less selective. Most studies on affinity labeling of F_1 have been conducted with analogues of adenine nucleotides modified on the adenosine moiety and therefore have brought information mainly on the catalytic sites (Russell et al., 1976; Wagenvoord et al., 1977; Lunardi et al., 1977; Hulla et al., 1978). Recently Budker et al. (1977) used ATP-γ-4-(*N*-2-chloroethyl-*N*-methylamino)benzylamidate, a bulky alkylating analogue, to retain ATP in its site.

The interest of the new analogue synthetized by Pal et al. (1975) is that the adenosine group is not modified and that the phosphoryl groups are replaced by an alkylating fluorosulfonylbenzoyl group (5′-fluorosulfonylbenzoyladenosine = FSBA),[1] the overall size of the molecule being very similar to that of ATP. As suggested by Colman et al. (1977), FSBA can reasonably be considered as an analogue of both ADP and ATP. It has been used for the stoichiometric labeling of adenine nucleotide specific sites of glutamate dehydrogenase (Pal et al., 1975), pyruvate kinase (Wyatt & Colman, 1977), ADP binding proteins of human platelets (Bennett et al., 1976), and phosphofructokinase (Mansour & Colman, 1978; Pettigrew & Frieden, 1978). The present work demonstrates that, due to the great specificity of the regulatory sites for the adenosine moiety, FSBA is a good analogue for the irreversible affinity labeling of both catalytic and regulatory sites of purified F_1-ATPase. In addition FSBA appears to be a good probe of conformational changes of the enzyme induced by its substrates.

Experimental Procedure

Materials. ADP and ATP were purchased from Boehringer Mannheim; their purity was monitored by high-pressure liquid chromatography using "Permaphase" ABX resin (Du Pont); the nucleotides were eluted by applying an exponential pH gradient made with 2.5 mM potassium phosphate, pH 4.5, and 100 mM potassium phosphate, pH 8.0 (Font & Vial, 1975, unpublished data). This technique showed that the ADP contained less than 1% ATP and that the ATP was contaminated by no more than 0.5% ADP. The [*carboxyl*-^{14}C]-*p*-aminobenzoic acid (35.6 mCi/mmol) used to begin the [*benzoyl*-^{14}C]FSBA synthesis was obtained from ICN Pharmaceuticals, Inc. All other chemicals were of reagent grade.

Preparation of Unlabeled and Labeled 5′-p-Fluorosulfonylbenzoyladenosine (FSBA). The unlabeled FSBA was prepared by the reaction of 4.2 mmol of adenosine with 6 mmol of *p*-fluorosulfonylbenzoyl chloride (Wyatt & Colman, 1977). The latter reactant was either purchased from Aldrich or synthesized in three steps, starting from *p*-aminobenzoic acid (Esch & Allison, 1978a). In both cases, the final products were obtained in yields similar to those described by the above authors: 67% and 27%, respectively. The product synthesized according to Esch & Allison (1978a) had the following properties. The NMR spectrum measured in deuterated dimethyl sulfoxide showed the characteristic peaks: δ 4.45 (2 protons), 4.75 (3), 6.1 (1), 7.1 (2), 8.45 (4), 8.7 (1), 8.9 (1), 9.5 (2), and the presence of 1 mol of the solvent hexamethylphosphoric triamide (δ 2.55, 18 protons) per mol of FSBA. When chromatographed, the product gave a single spot which migrated together with the sample kindly given by Dr. R. Colman; silica gel F-254 thin-layer chromatography plates were used with a solvent system consisting either of methyl ethyl ketone–acetone–water (65:20:15), R_f 0.83, or methanol–chloroform (15:85), R_f 0.75. The ultraviolet absorption spectrum of ethanolic FSBA solution showed two maxima: at 232 nm (ϵ 1.90×10^4 cm^{-1} M^{-1}) and 259 nm (ϵ 1.41×10^4 cm^{-1} M^{-1}). The melting point was 146 °C. All these criteria are in total agreement with the results of Wyatt & Colman (1977). However, it was found that the solubility of the product in ethanol was much lower (0.3 mM) when it contained 1 mol of hexamethylphosphoric triamide per mol than when it was recrystallized with dimethylformamide (7–8 mM) (Colman et al., 1977).

Radioactive FSBA was prepared by the addition of 500 μCi of [*carboxyl*-^{14}C]-*p*-aminobenzoic acid to 1 mmol of the nonlabeled *p*-aminobenzoic acid. The four-step synthesis of [*benzoyl*-^{14}C]FSBA was conducted as described by Esch & Allison (1978a). The specific radioactivity of the product (0.27 mmol) was 0.46 mCi/mmol. To control the specificity of the labeling, a sample was chromatographed and the plate was cut into 5-mm bands; each one was eluted by 0.4 mL of dimethyl sulfoxide and centrifuged; 0.2 mL of the supernatant was used for the measurement of the radioactivity by liquid scintillation. It was found that at least 93% of the added label was recovered in the spot of [*benzoyl*-^{14}C]FSBA, R_f value of 0.83, when the methyl ethyl ketone–acetone–water system was used.

The labeled and unlabeled FSBA produced the same inhibition of the enzyme.

Preparation of F_1-ATPase. Pig heart mitochondria were obtained at 0–4 °C as previously described (Gautheron et al., 1964). F_1 was purified by a procedure derived from that of Senior & Brooks (1970), as reported earlier (Di Pietro et al., 1975). The last step of the preparation, which consists of heating F_1, was omitted since it suppresses the cooperativity (Godinot et al., 1975). The enzyme had a specific activity of 90–110 units/mg of protein (1 unit = 1 μmol of ATP hydrolyzed per min) and was stored as a suspension in 40 mM Tris–H_2SO_4–1 mM EDTA–2 mM ATP–0.2 M potassium phosphate buffer, pH 7.5, containing 60% saturated ammonium sulfate. Just before use, an aliquot was centrifuged at 4 °C for 5 min at 9000*g* in an "Eppendorf Zentrifuge 3 200" and then washed in 40 mM Tris–H_2SO_4–1 mM EDTA buffer, pH 7.5 (Tris–EDTA buffer), or in 0.2 M potassium phosphate–5 mM EDTA buffer, pH 7.5 (phosphate–EDTA buffer), containing 60% saturated ammonium sulfate. The pellet was dissolved in one of the above buffers and incubated with the inhibitor. The molecular weight for F_1 was taken as 391 000 (Di Pietro et al., 1975).

Reaction of FSBA with F_1. In preliminary experiments, dimethylformamide was used to dissolve FSBA; however, as it was found to inhibit F_1-ATPase activity (62% inhibition was produced by 9% dimethylformamide in phosphate–EDTA buffer for 150 min), dimethyl sulfoxide, which did not inhibit, was used in the experiments described. The FSBA-induced inhibition of ATPase activity was the same in both solvents.

The reversible inhibition was measured kinetically at 30 °C by adding FSBA directly to the ATP hydrolysis medium which contained 42 mM Tris–maleate buffer, pH 8.0 (0.8 mL final volume), and the indicated concentrations of FSBA in dimethyl

[1] Abbreviations used: FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; Tris–EDTA buffer, 40 mM Tris–H_2SO_4–1 mM EDTA, pH 7.5; phosphate–EDTA buffer, 0.2 M potassium phosphate–5 mM EDTA, pH 7.5.

sulfoxide and of ATP and $MgSO_4$, the concentrations of ATP and $MgSO_4$ being always in the ratio 1:1 during measurements of initial velocities. The reaction was initiated by adding a 30-μL aliquot containing 2 μg of F_1 in Tris–EDTA buffer and stopped after 1 min by 75 μL of ice-cold, 50% trichloroacetic acid. The inorganic phosphate released was measured by the colorimetric method of Sumner (1944).

The irreversible inactivation was studied by incubating for long periods 40 μg of F_1 with 0.09–0.57 mM FSBA in 200 μL of Tris–EDTA or phosphate–EDTA buffer at 30 °C. These buffers were preferred to the barbital–KCl buffer used by other authors (Pal et al., 1975; Wyatt & Colman, 1977) since the enzyme activity measured in the latter buffer was only about 40% of that obtained in the former buffers. The FSBA-induced inhibition of F_1 in Tris–EDTA buffer was very similar to that obtained in the barbital–KCl buffer. To study irreversible inactivation as a function of time, 5-μL aliquots of the mixture were assayed for residual ATPase activity by a recording spectrophotometric method, using pyruvate kinase and lactate dehydrogenase as auxiliary enzymes and measuring the rate of NADH disappearance (Pullman et al., 1960). The 42 mM Tris–maleate buffer, pH 8.0, was also used in this assay with 3.3 mM ATP and 3.3 mM $MgSO_4$. For both reversible inhibition and irreversible inactivation, control values were determined using equal volumes of the solvent dimethyl sulfoxide.

To determine the incorporation of radioactive FSBA in the enzyme during the irreversible inactivation, F_1 was incubated in Tris–EDTA buffer containing 0.57 mM [*benzoyl*-^{14}C]-FSBA. At the specified times, the radioactivity retained on the enzyme was measured either after Sephadex G-25 M gel filtration or after precipitating the proteins with ethanol. In the first case, 1 mL of incubation medium contained 4 mg of F_1 and 100-μL aliquots were precipitated by 150 μL of saturated ammonium sulfate, diluted tenfold with Tris–EDTA buffer containing 60% saturated ammonium sulfate, and centrifuged at 9000*g* for 5 min. The pellets were dissolved in 170 μL of Tris–EDTA buffer and were chromatographed on Sephadex G-25 M columns (Pharmacia) equilibrated with the same buffer. The dimensions of the columns were 1.5 × 5.2 cm, the flow rate was 0.6 mL/min, and the fraction volume was 0.4 mL. The enzyme was generally eluted in fractions 7 to 10 and was located by measuring the ATPase activity of 2-μL aliquots using the spectrophotometric assay. For each fraction, the protein content (Lowry et al., 1951) and the radioactivity were measured using 100 and 250 μL, respectively. In the second case, 1 mL of the incubation mixture contained 1 mg of F_1 and 100-μL aliquots were precipitated by 10 volumes of ethanol (Senior, 1975) and centrifuged at 9000*g* for 5 min. The pellets were dissolved in 100 μL of Tris–EDTA buffer and reprecipitated twice. The final pellets were dissolved in 200 μL of 1 N NaOH of which 40 μL was used for protein measurement and 140 μL was counted. The residual ATPase activity could only be measured before the addition of alcohol since the precipitation denatured the enzyme.

As, at each indicated time, the radioactivity bound to the enzyme, the amount of protein and the residual activity were known, a relationship between the number of moles of FSBA bound per mole of F_1 and the percentage of the initial enzyme activity could be determined.

Results

Incubation of F_1 at pHs between 7.5 and 8.0 with FSBA revealed that the adenine nucleotide analogue inhibited the ATPase activity of the enzyme. An irreversible inactivation of F_1 was observed and increased with the length of the incubation time. However, at the very beginning of the incubation it was negligible with respect to the reversible inhibition that immediately appeared. Classical kinetic data could be obtained for incubation times as short as 1 min.

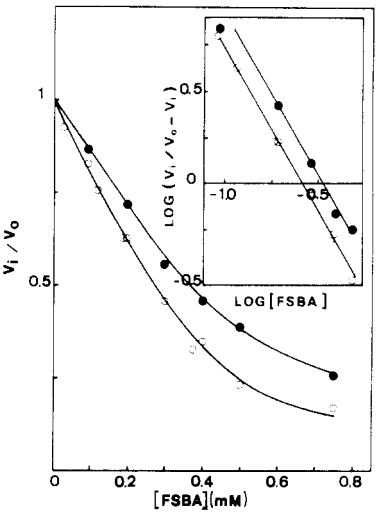

FIGURE 1: Reversible inhibition of F_1-ATPase activity as a function of FSBA concentration. The enzyme was incubated in 0.8 mL of 42 mM Tris–maleate buffer, pH 8.0, for 1 min with 40 μL of FSBA at various concentrations in dimethyl sulfoxide, in the presence of the indicated ATP concentrations. The residual activity V_i was measured by the colorimetric method (see Experimental Procedure). The initial activity V_0 was 75 units/mg with 1.1 mM ATP (●) or 43 units/mg with 0.27 mM ATP (○). Insert: Determination of the Hill number for FSBA during the reversible inhibition of ATPase activity. The data were plotted according to Hill (1910).

Kinetics of Reversible Inhibition of F_1-ATPase Activity by FSBA. When the ATPase activity of F_1 was measured for 1 min in Tris–maleate buffer, pH 8.0, with increasing concentrations of FSBA in dimethyl sulfoxide, the enzyme activity diminished as shown in Figure 1; in control experiments made with dimethyl sulfoxide in the absence of FSBA, there was no change in the ATPase activity. Increasing the ATP concentration of the medium partly protected against the FSBA-induced inhibition; 50% inhibition was obtained, for example, at 0.27 mM FSBA for 0.27 mM ATP and at 0.36 mM FSBA for 1.1 mM ATP. If these results were analyzed according to Dixon (1953) to determine the type of inhibition, nonlinear plots were obtained with an upward concave curvature (not shown here) indicating a positive cooperativity between the ATP and FSBA sites. The determination of the Hill number for FSBA (insert, Figure 1) gave a value of 1.8 ± 0.2 for ATP concentrations between 0.22 and 2.5 mM. This inhibition was completely reversible upon 100-fold dilution, if the incubation of F_1 with FSBA did not exceed 1 min. Therefore it was concluded that F_1 has at least two reversible binding sites for FSBA.

When the Hill number for ATP was determined (plot not shown here) in the same conditions (ten different ATP concentrations from 0.22 to 2.5 mM), the value varied from 1.05 in the absence of FSBA to 2.3 for 0.75 mM FSBA.

Kinetic Studies of the Irreversible Inactivation of F_1 by FSBA. The irreversible inactivation was followed by incubating F_1 in Tris–EDTA buffer with FSBA for 150 min at 30 °C. At the indicated times, aliquots were diluted 100-fold and assayed for ATPase activity. Controls without FSBA, in the presence or absence of the solvent dimethyl sulfoxide, showed no change in activity. A semilogarithmic plot of V_i/V_0 against time (Figure 2) reveals a biphasic rate of inactivation with a rapid reaction and a slower one that leads to complete

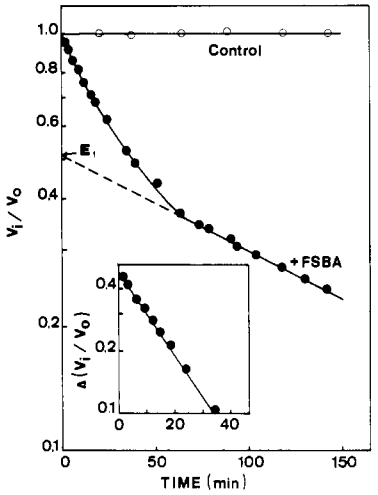

FIGURE 2: Irreversible inactivation of F_1 by FSBA. The enzyme (40 μg) was incubated at 30 °C in 200 μL of Tris–EDTA buffer with 10 μL of 12 mM FSBA (0.57 mM final concentration). At the times indicated, a 5-μL aliquot was assayed for residual ATPase activity V_i by the spectrophotometric method described above; initial activity V_0 was 104 units/mg; E_1 represents the fractional activity remaining after the rapid phase (E_1 = 0.51); k_2 was determined from the final slope. Insert: Determination of the constant k_1 for the rapid phase. Subtracting the observed values on the curve from the corresponding values on the extrapolated slope gives the corresponding $\Delta(V_i/V_0)$ values; k_1 is equal to the slope of the semilogarithmic plot of $\Delta(V_i/V_0)$ against time (Ray & Koshland, 1961).

inhibition of the enzyme. The slower phase was found to be linear at least up to a residual activity of 10%. This behavior, according to Ray & Koshland (1961), could be attributed to the presence of partially active enzyme species that would correspond to the fixation of FSBA at one type of site and completely inactive species corresponding to the fixation of FSBA to another type of site. The individual rate constants can be determined graphically: "the final slope gives the latter rate constant k_2 and subtraction of the values along the extrapolated slope from the observed values gives the former constant k_1" (Ray & Koshland, 1961). One can directly determine a k_2 value of 5.8×10^{-3} min^{-1} from the slope of the curve of Figure 2 between 60 and 150 min and a k_1 value of 43×10^{-3} min^{-1} (insert, Figure 2). If F_1 was incubated with 0.46 mM FSBA for 40 min, which approximately corresponds to the end of the rapid phase of inactivation, the K_m for ATP was not modified as compared with that of the untreated enzyme (0.26 mM); an inhibition of 41% of V_m was observed. This result may indicate that the rapid phase of inhibition diminishes the catalytic activity of the enzyme without modifying the binding of ATP.

The values of k_1 and k_2 and the time taken to reach 50% inhibition by 0.57 mM FSBA ($t_{1/2}$) were studied in the presence of various effectors (Table I). The constant k_1 appeared insensitive to all tested effectors (2 mM ATP, 2 mM ADP, 8 mM Mg^{2+}, 20% glycerol, 20% glycerol + 2 mM ATP) in Tris–EDTA buffer; only 0.2 M phosphate was able to increase k_1 by twofold at the maximum except in the presence of glycerol, the increase being proportional to phosphate concentration and independent of the ionic strength of the medium. In contrast, k_2 was very sensitive to any kind of effector. The values obtained in phosphate–EDTA buffer were always higher than in Tris–EDTA buffer. ATP or ADP (2 mM) decreased k_2, while 8 mM Mg^{2+} slightly increased it; the presence of 20% glycerol strongly decreased the value of k_2. In every case, k_1 was much higher than k_2. Since k_1 was not affected in Tris–EDTA buffer, obviously the important changes in $t_{1/2}$ observed in this case reflect the variations of

Table I: Effects of Buffers and Effectors on the Kinetic Parameters of FSBA-Induced Inactivation of F_1[a]

incubation medium	$t_{1/2}$[b] (min)	$k_1 \times 10^3$ (min^{-1})	$k_2 \times 10^3$ (min^{-1})
Tris–EDTA buffer	34	43	5.8
+ 2 mM ATP	41	43	3.7
+ 2 mM ADP	80	43	2.9
+ 8 mM Mg^{2+}	31	43	6.7
+ 20% glycerol	≃400	43	1.2
+ glycerol + ATP	≃400	43	1.2
phosphate–EDTA buffer	10	84	15.3
+ 2 mM ATP	16	79	8.6
+ 20% glycerol	33	43	3.3
+ glycerol + ATP	42	43	2.4

[a] Conditions were the same as in Figure 2 except that the indicated effectors were added and phosphate–EDTA buffer replaced Tris–EDTA buffer when specified. FSBA concentration was 0.57 mM and the constants k_1 and k_2 were calculated as described in Figure 2. [b] $t_{1/2}$ represents the time taken to reach 50% of the total inhibition.

k_2. However, the extent of the inhibition $1 - E_1$ (cf. Figure 2), due to the rapid phase of inactivation characterized by k_1, depends also on the number of available sites and is taken into account in the measured $t_{1/2}$.

Effects of FSBA Concentration. The two phases of the inactivation curve were studied by incubating the enzyme with FSBA concentrations varying from 0.09 to 0.57 mM. In this range, the k_1 value was always independent of the FSBA concentration. It is possible that the lowest FSBA concentration used is already saturating for the binding of the analogue to the type of sites corresponding to the constant k_1. However, it would be difficult to test precisely lower concentrations of FSBA as the induced inhibition would be too limited. On the contrary, when studied in Tris–EDTA buffer, the pseudo-first-order rate constant k_2 increased with the FSBA concentration. The plot of the value of this rate constant against the reagent concentration was not linear, but revealed saturation kinetics. This type of behavior has been interpreted by Wyatt & Colman (1977) in the case of pyruvate kinase as indicative of a reversible binding to the considered site prior to irreversible inactivation, according to the following reaction:

$$E + FSBA \overset{K_D}{\rightleftharpoons} [E\text{–}FSBA] \xrightarrow{k_2} [E\text{–}FSBA]^*$$

where [E–FSBA] is the reversible form and [E–FSBA]* is the irreversible inactivated form of the complex and K_D is the dissociation constant for the [E–FSBA] complex. The apparent rate constant $k_{2(app)}$ for the inactivation can be expressed as

$$k_{2(app)} = \frac{k_2}{1 + (K_D/[FSBA])}$$

The reciprocal plot (Figure 3A) gives an extrapolated k_2 value of 8.2×10^{-3} min^{-1} when the enzyme is saturated by FSBA and a dissociation constant for the reversible enzyme–ligand complex of 0.23 mM. This K_D value is quite comparable to the K_m of the enzyme for its substrate ATP (0.26 mM).

When the irreversible inactivation of F_1 was studied in phosphate–EDTA buffer instead of Tris–EDTA buffer, the inhibition was much stronger. The two pseudo-first-order rate constants k_1 and k_2 were much higher, as shown above (Table I), but both were independent of FSBA concentration in the same range studied. However, the value $1 - E_1$, which may

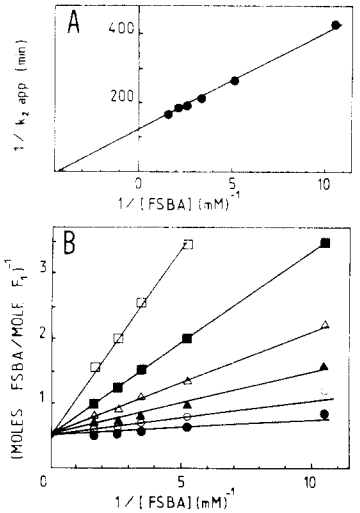

FIGURE 3: Dependence of the second phase of inactivation on FSBA concentration. (A) Dependence of the pseudo-first-order rate constant on FSBA concentration; the incubation and assay conditions were the same as in Figure 2 except that the FSBA concentration was varied from 0.09 to 0.57 mM; (B) evaluation of the number of moles of FSBA bound during the second phase of inactivation as a function of the FSBA concentration; inactivation curves were obtained in Tris–EDTA buffer as in A. At the times indicated during the second phase, the residual ATPase activity was converted into FSBA incorporation using Figure 5 as a standard curve; from this value was subtracted that of the corresponding first phase. The times selected for the calculations were: 40 min (□); 60 min (■); 80 min (△); 100 min (▲); 120 min (○); and 150 min (●).

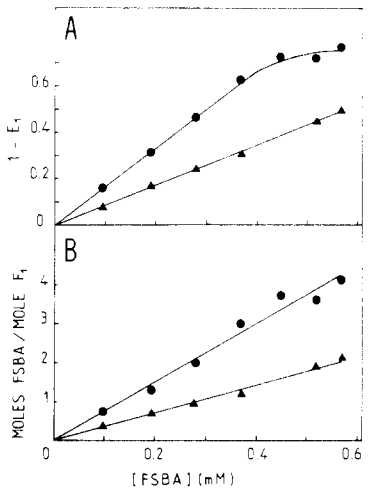

FIGURE 4: Dependence of the first phase of inactivation on the FSBA concentration. (A) The effect on the extent of inactivation $1 - E_1$ was measured by incubating F_1 at various FSBA concentrations from 0.09 to 0.57 mM in the Tris–EDTA buffer (▲) or in the phosphate–EDTA buffer (●); $1 - E_1$ was determined graphically as shown in Figure 2. (B) The number of moles of FSBA bound per mole of F_1 was evaluated, in both Tris–EDTA (▲) and phosphate–EDTA (●) assuming that the incorporation is correlated to the inhibition of ATPase activity as shown in Figure 5; the theoretical number of moles of FSBA bound per mole of F_1 at the different FSBA concentrations was determined from the inhibition data obtained in A.

be indicative of the fractional inactivation due to the rapid phase, increased with FSBA concentration (Figure 4A). This increase in $1 - E_1$ should only be attributable to a larger number of sites available for the rapid phase. In Tris–EDTA buffer, the increase of $1 - E_1$ could be attributed either to the change in k_2 (cf. Figure 3A) or to an increased number of sites of the rapid phase or to both. The addition of 2 mM ADP or 20% glycerol decreased $1 - E_1$ by 25% and 50%, respectively, while ATP had no effect.

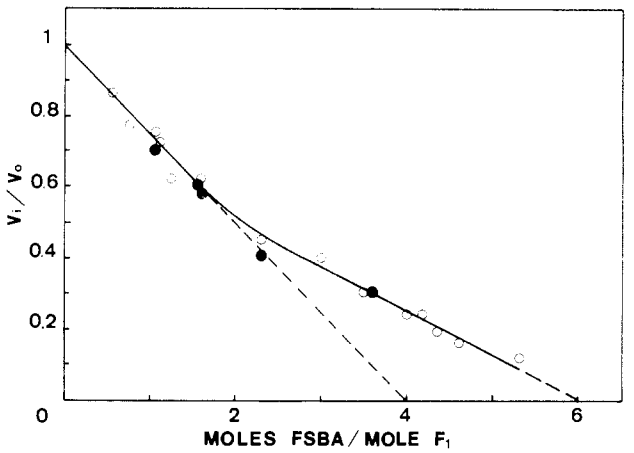

FIGURE 5: Relationship between the incorporation of radioactive FSBA and the extent of F_1 inactivation. F_1 was incubated at 30 °C in Tris–EDTA buffer with 0.57 mM FSBA. At intervals over 300 min, aliquots were withdrawn for the measurement of residual ATPase activity by the spectrophotometric method, of the protein content and of the radioactivity incorporated, using gel filtration (●) or ethanol precipitation (○) as described in the Experimental Procedure.

To test whether the inactivating effect of FSBA was specifically related to its adenosine moiety, the effects of *p*-fluorosulfonylbenzoyl chloride were studied on the ATPase activity. The incubation of F_1 in Tris–EDTA buffer with 0.57 mM *p*-fluorosulfonylbenzoyl chloride gave only 2% and 5% inhibition when an identical FSBA concentration gave respectively 50% and 75% inhibition for the same incubation times. In phosphate–EDTA buffer, the specificity of the adenosine moiety was lower since 12% and 21% inhibition, respectively, were observed with *p*-fluorosulfonylbenzoyl chloride as compared with 50% and 80% inhibition observed with FSBA. This binding of the *p*-fluorosulfonyl benzoyl moiety was completely masked when 20% glycerol was added to the phosphate–EDTA buffer.

Incorporation of Radioactive FSBA in F_1. To evaluate the amount of FSBA irreversibly bound during the inactivation, F_1 was incubated in Tris–EDTA buffer with 0.57 mM [*benzoyl*-^{14}C]FSBA and the radioactivity incorporated into the enzyme at different times was correlated to the corresponding residual activity. The same results were obtained if the unbound ligand was separated from the enzyme by ammonium sulfate precipitation followed by gel filtration or by three successive ethanol precipitations (Figure 5). A biphasic relationship was obtained between the loss of ATPase activity and the number of moles of FSBA bound per mole of F_1. Extrapolation from the curve to 100% inhibition of ATPase activity corresponds to the binding of 6 mol of FSBA per mol of F_1. Extrapolation of the first rapid phase of binding to 100% inhibition would give four binding sites for FSBA per mol of F_1. This biphasic curve also indicates the presence of two kinds of binding sites.

It can be assumed that the number of moles of FSBA incorporated into F_1 is always correlated to the inhibition of the ATPase activity, following the relationship shown in Figure 5. It is then possible to evaluate the theoretical number of moles of FSBA bound to F_1 from the kinetic data of F_1 inactivation obtained in different conditions. For example, varying the FSBA concentration as in Figure 4A would give the results presented in Figure 4B which show that the number of moles of FSBA bound per mole of F_1 during the rapid phase of inactivation increases more rapidly in phosphate–EDTA buffer than in Tris–EDTA buffer; this number is decreased by ADP and glycerol but is not modified by ATP. Table II

Table II: Effects of Buffers and Effectors on the Extent of the Rapid Phase of Inactivation and on the Radioactive FSBA Incorporation[a]

incubation medium	$1-E_1$[b]	mol of FSBA/mol of F_1: theor value[c]	mol of FSBA/mol of F_1: direct binding measurement[d]
Tris-EDTA buffer	0.49	2.2	2.5
+ 2 mM ATP	0.49	2.2	4.5
+ 2 mM ADP	0.38	1.5	1.7
+ 20% glycerol	0.24	1.2	1.1
phosphate-EDTA buffer	0.78	4.3	4.4
+ 2 mM ATP	0.73	3.8	13.9
+ 20% glycerol	0.26	1.1	1.1
+ ATP + glycerol	0.25	1.0	8.3

[a] Conditions were the same as in Table I for incubation and residual activity assay. FSBA concentration was 0.57 mM. [b] In each case, the irreversible inactivation of F_1 was followed as a function of time and E_1 was determined as shown in Figure 2. [c] Each value of E_1 is a measure of V_i/V_0 which can theoretically be related to the moles of FSBA bound per mole of F_1 as in Figure 5. [d] At the time corresponding to a residual activity equal to E_1, the binding of radioactive FSBA was measured by ethanol precipitation as described in the Experimental Procedure.

demonstrates that the above assumption was justified in most cases. In each experiment, the irreversible inactivation of F_1 was followed as a function of time, E_1 was graphically determined, and $1 - E_1$ was calculated. At the time corresponding to E_1, the theoretical number of moles of FSBA bound per mole of F_1 was determined using Figure 5 as a standard curve; at the same time the actual number of moles of radioactive FSBA bound was directly measured experimentally by the ethanol precipitation technique. Table II indicates that the theoretical values, as calculated by the percentage of inhibition, are always in good agreement with the experimental values except in the presence of ATP. In the latter case, about 14 mol of FSBA is bound indicating that more binding sites become accessible, which are independent of the ATP hydrolytic sites. In the presence of all other effectors, the number of available sites for FSBA binding was directly correlated to the extent of inhibition of enzyme activity; in this way, ADP and glycerol which protected against enzyme inactivation also decreased the FSBA binding.

The same principle of calculation has been used to evaluate the number of sites implicated in the slow phase of inactivation. For this purpose, the inactivation of F_1 was studied for 150 min in Tris–EDTA buffer in the presence of various FSBA concentrations. For each concentration, the value of E_1 was obtained graphically by extrapolation and $1 - E_1$ was calculated. The number N_1 of moles of FSBA bound per mole of F_1 corresponding to $1 - E_1$, that is to the rapid phase, was determined using Figure 5 as a standard curve. In addition at each indicated time of the slow phase, the V_i/V_0 ratio was measured and the total corresponding number N_2 of moles of FSBA bound per mole of F_1 was deduced from Figure 5. The difference $N_2 - N_1$ gives the estimated number of moles of FSBA bound during the slow phase. For example, when the FSBA concentration was 0.57 mM, $E_1 = 0.51$ (Figure 2) and for $1 - E_1 = 0.49$, $N_1 = 2.1$ mol of FSBA bound (Figure 5). At the time = 80 min, $V_i/V_0 = 0.335$ (Figure 2), and, for this value of V_i/V_0, $N_2 = 3.35$ mol of FSBA bound (Figure 5); $N_2 - N_1 = 1.25$ mol of FSBA bound during the second phase. When the same calculation was made at different times for each FSBA concentration, it gave results which exhibit saturation kinetics; the reciprocal plots (Figure 3B) indicate that a maximal number of 2 mol of FSBA could bind per mol of F_1 during the slow phase of inactivation, irrespective of the incubation time.

Discussion

The adenine nucleotide analogue 5′-*p*-fluorosulfonylbenzoyladenosine serves as a good affinity label for the mitochondrial ATPase F_1. It reacts both at the regulatory and the hydrolytic sites, inhibiting and inactivating the enzyme. A limited number of moles of the analogue can be bound on the enzyme; both ADP and ATP partially prevent the observed inhibitions induced by FSBA and affect the binding of the analogue. The inhibitions are very dependent on the adenosine moiety of the molecule in Tris–EDTA buffer; however, in the presence of phosphate, an additional unspecific inhibition due to the *p*-fluorosulfonylbenzoyl moiety is also observed. The final binding of the analogue is irreversible and, therefore, the analogue is a good affinity label. However, the kinetic data indicate that a reversible association occurs before the irreversible reaction. Indeed the initial inhibition is completely reversed and the saturation kinetics of the pseudo-first-order rate constant calculated in the second phase of inactivation also indicate the formation of a reversible complex prior to the irreversible binding. The dissociation constant of this reversible complex is identical with the Michaelis constant of the enzyme for ATP and therefore reflects the binding of the analogue to the catalytic ATPase site(s).

In a previous study, Pal et al. (1975) showed that FSBA could bind specifically to a regulatory NADH site of bovine liver glutamate dehydrogenase. Wyatt & Colman (1977) found two types of binding sites in rabbit muscle pyruvate kinase, one at least being a Mg–ADP site. The pseudo-first-order rate constants of inactivation of these enzymes by FSBA and those measured in the present work with mitochondrial F_1-ATPase are all very similar which could indicate that the same species of amino acids are involved in the reaction with FSBA at the binding sites. Colman et al. (1977) suggested that the sulfonyl fluoride moiety acts as an electrophilic agent in covalent reactions with several classes of amino acids, including serine, tyrosine, lysine, histidine, and cysteine. The lack of cysteine residues directly involved in the hydrolytic site of F_1 is well documented (Farron & Racker, 1970; Senior, 1973; Godinot et al., 1975, 1977; Pedersen, 1976), although some results suggest the presence of thiols related to the anion regulatory site (Pedersen, 1976). Arguments have been presented for the absence of histidine (Godinot et al., 1979); on the contrary, experimental data are in favor of the presence of tyrosine (Senior, 1973; Ferguson et al., 1974), of lysine (cf. review by Kozlov & Skulachev, 1977), of arginine (Marcus et al., 1976; Frigeri et al., 1977), and of carboxyl groups (Kozlov & Skulachev, 1977; Pougeois et al., 1978). It is therefore likely that FSBA reacts with some of these amino acids in the catalytic site(s). In contrast, little is known about the amino acids present in the regulatory sites. The use of FSBA could permit, in further investigations, the labeling of the regulatory as well as the catalytic sites and the determination of their structure by peptide map analysis.

The study of the FSBA-induced irreversible inactivation of F_1 and the binding of radioactive FSBA demonstrated the existence of two types of sites. During the rapid phase of inactivation, FSBA must first be bound reversibly with a very high affinity, since the rate constant of inactivation is already maximal at the lowest FSBA concentration tested (90 μM), and then it must react irreversibly with a limited rate. Such high affinity is in favor of the binding of FSBA to the ADP regulatory sites. Indeed, on one hand, FSBA has been shown

to bind very efficiently to ADP sites in the case of ADP binding proteins of human platelets (Bennett et al., 1976), rabbit muscle pyruvate kinase (Wyatt & Colman, 1977), and phosphofructokinase from sheep heart (Mansour & Colman, 1978) and from rabbit muscle (Pettigrew & Frieden, 1978); on the other hand, the "tight" site of ADP binding to F_1 has a very low K_D (0.28 μM) (Hilborn & Hammes, 1973) compared with the "loose" site (47 μM) and to the Michaelis constant of F_1 for ATP (0.26 mM). Besides the "tight" site is very specific for ADP (Hilborn & Hammes, 1973; Schuster et al., 1975; Pedersen, 1975). The protection by ADP against inactivation and the kinetics of the reversible FSBA-induced inhibition of F_1 also indicate that FSBA binds to the ADP sites. In the latter case, the analogue increases, even more than ADP, the Hill number of F_1 for ATP; when ADP concentration was varied from 0 to 1.2 mM, the Hill number for ATP increased from 1.0 to 1.34 (Godinot et al., 1975) while 0.75 mM FSBA brought this Hill number for ATP to 2.3. What happens during the first rapid phase may induce the formation of partly active species in which V_m is diminished and K_m for ATP remains unchanged.

The slower phase of inactivation must correspond to the binding of FSBA to the ATPase catalytic site. Its dependence on FSBA concentration showing saturation kinetics allows the determination of a K_D for the reversible binding of FSBA to F_1 of 0.23 mM which compares quite well with the K_m for ATP (0.26 mM). This demonstrates that FSBA binds at the same sites as ATP. A maximal number of 6 mol of FSBA is bound per mol of F_1 when the enzyme is completely inhibited. Of these six sites, a maximum of two can be related to the slow phase of inactivation. It is tempting to correlate these two binding sites (which are believed to belong to the hydrolytic site) to the two sites labeled by 8-azido-ATP (Wagenvoord et al., 1977) with a complete inhibition of ATPase activity. Very recently, Hulla et al. (1978) and Höckel et al. (1978) found a maximum of 6 ± 1 tight binding sites of nucleotides per mol of bacterial F_1-ATPase; from these sites, at least two could bind covalently the ATP analogue 6-[(3-carboxy-4-nitrophenyl)thio]-9-β-D-ribofuranosylpurine 5′-triphosphate. It is possible that in a similar way FSBA also binds to the tight binding sites. Our results concerning the reversible inhibition of F_1 agree with the presence of 2 catalytic sites to which FSBA could bind; indeed a Hill number of nearly 2 is obtained for FSBA when the inhibition is studied in a concentration range that modifies the rate constant of the slow phase.

This study has also shown that effectors can induce important conformational changes of the enzyme. In the presence of ATP, the rate of inactivation diminishes, but the number of FSBA binding sites unexpectedly increases. A similar increase in FSBA binding to pyruvate kinase was obtained by Wyatt & Colman (1977) when studying the protective effect of phosphoenol pyruvate upon enzyme inactivation; the above authors also concluded that a conformational change of pyruvate kinase was induced by its substrate. The present work confirms the previously observed conformational changes of F_1 detected by the greater availability of titrable thiol groups induced by ATP (Godinot et al., 1975, 1977). The presence of phosphate, another substrate which binds to F_1 (Penefsky, 1977), also modifies the reaction of FSBA inducing another enzyme conformation. It is likely that phosphate exposes some hydrophobic area since the enzyme becomes very sensitive to hydrophobic compounds such as dimethylformamide or 5′-*p*-fluorosulfonylbenzoyl chloride. Addition of 20% glycerol completely protects the enzyme against this kind of inactivation. In both Tris–EDTA and phosphate–EDTA buffers, glycerol also protects very efficiently against the specific FSBA-induced inactivation of F_1 by lowering the analogue binding. It is known that high concentrations of glycerol protect F_1 against cold-dissociation (Penefsky & Warner, 1965) and allow a better reassociation of F_1 subunits to give an active enzyme either from cold-denatured preparation (Rosing et al., 1975) or from pure isolated subunits (Futai, 1977). Garret & Penefsky (1975) found that, after removal of all nucleotides by 50% glycerol, F_1 remained stable for long periods. Thus glycerol may replace the nucleotides involved in the overall structure of F_1.

Furthermore, Shaughnessy et al. (1977) found that glycerol seems to increase the ionization of the phenolic groups of tyrosines; therefore a direct effect on tyrosine residues related to the catalytic sites could be considered. Such an effect could explain the important protective effect of glycerol during the slow phase of inactivation which is assumed to be related to the catalytic sites of F_1.

After the completion of this manuscript, Esch & Allison (1978b) reported that FSBA binds to the catalytic sites. They observed a very stable covalent binding to the β subunit and showed that a tyrosine residue was present in the active site. They also found less stable binding to the α subunit. The question remains whether or not this labeling of the α and β subunits corresponds to binding at the regulatory and catalytic sites. Further work is in progress in our laboratory to elucidate the exact location of these sites.

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Mitochondrial Creatine Kinase. Physical and Kinetic Properties of the Purified Enzyme from Beef Heart†

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ABSTRACT: An improved method for the purification of the mitochondrial isozyme of creatine kinase is reported. Studies of the properties of the purified enzyme from beef heart reveal pH optima of 8 and 6–6.5 for the forward and reverse reactions, respectively. The apparent K_m values for Mg-ATP and creatine are 0.056 and 4.5 mM and for Mg-ADP and phosphocreatine are 0.015 and 0.31 mM. The smallest active form of the enzyme is a dimer of 64 000 daltons. Two active forms are found in solution, one with a slightly cathodal mobility on cellulose polyacetate strip electrophoresis and a molecular weight of about 64 000 and another with a greater cathodal mobility on electrophoresis and with a molecular weight at least three times larger. The two forms can be separated by molecular seive chromatography but interconvert in solution, with the extent of the conversion dependent upon protein concentration and on the presence of a reducing agent. Intermolecular hybrids between subunits of the heart mitochondrial isozyme and subunits of other beef creatine kinase isozymes were not formed upon disruption and reannealing. The results of these studies are consistent with the hypothesis that mitochondrial creatine kinase is especially adapted to the formation of phosphocreatine in the mitochondrion and may have a very different structure from that of the MM, MB, and BB isozymes.

The enzyme creatine kinase (EC 2.7.3.2), which catalyzes the transfer of phosphate from ATP to creatine to produce ADP and phosphocreatine, has long been assumed to be involved in the regeneration of ATP in association with contractile or transport systems (Kuby & Noltman, 1962; Watts, 1973). Several different isozymes of creatine kinase have been isolated from mammalian tissues. The best characterized of these are commonly referred to as BB (CK-1, brain), MB (CK-2, hybrid, present in mammalian heart), and MM (CK-3, muscle). These enzymes have different kinetic parameters (Eppenberger et al., 1967; Dawson et al., 1967) and different amino acid compositions (Watts, 1973) and can be separated electrophoretically at pH 8.8 (Burger et al., 1964; Eppenberger et al., 1964). Treatment of a mixture of the MM and BB types

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