Dimensional Probes of the Enzyme Binding Sites of Adenine Nucleotides. Interaction of *lin*-Benzoadenosine 5′-Di- and Triphosphate with Mitochondrial ATP Synthetase, Purified ATPase, and the Adenine Nucleotide Carrier†

Raymond F. Kauffman, Henry A. Lardy, Jorge R. Barrio, Maria del Carmen G. Barrio, and Nelson J. Leonard*

ABSTRACT: The adenine nucleotide analogues *lin*-benzoadenosine 5′-diphosphate and *lin*-benzoadenosine 5′-triphosphate were found to be substrates for phosphorylation by submitochondrial particles and for hydrolysis by the purified mitochondrial ATPase. A technique is described which simplifies the kinetics of phosphorylation by submitochondrial particles. Substrate inhibition by P_i became apparent with *lin*-benzoadenosine 5′-diphosphate as the substrate for phosphorylation in the particles. The purified mitochondrial ATPase was inhibited more potently by *lin*-benzoadenosine 5′-diphosphate than by ADP. The fluorescence of *lin*-benzoadenosine 5′-diphosphate was strongly quenched by the purified mitochondrial ATPase. With intact mitochondria *lin*-benzoadenosine 5′-diphosphate was a poor acceptor for oxidative phosphorylation. Both the rate and extent of $^{32}P_i$ incorporation into organic phosphates were enhanced only slightly by *lin*-benzoadenosine 5′-diphosphate, and this enhancement was completely sensitive to ethylenediaminetetraacetate but not to fluoride. The ADP-stimulated respiration rate and the P/O ratio for these mitochondria were not affected by ethylenediaminetetraacetate. *lin*-Benzoadenosine 5′-di- or triphosphate displaced only minute amounts of radioactivity from intact mitochondria loaded with [^{14}C]ADP. These data indicate that *lin*-benzoadenosine 5′-di- and triphosphates displayed little, if any, activity as substrates for the adenine nucleotide carrier. The possibility that nucleoside diphosphokinase in the intermembrane space transferred [^{32}P]-phosphoryl groups from endogenous ATP to *lin*-benzoadenosine 5′-diphosphate is discussed.

One approach to understanding the mechanism of mitochondrial oxidative phosphorylation is to examine the chemical composition and dimensional restrictions of the site(s) involved in catalysis. In the absence of X-ray crystallographic data, this information must be obtained by other studies such as chemical modification and the use of analogues of natural ligands. Although construction of a model for the catalytic site(s) of the coupling factor is not possible with the data presently available, knowledge in this area is progressing (for reviews, see Pedersen, 1975; Kozlov & Skulachev, 1977).

Fluorescent "stretched-out" analogues of adenine nucleotides (*lin*[1]-benzoadenosine 5′-monophosphate, 5′-diphosphate, and 5′-triphosphate) and their biological activity have recently been described (Scopes et al., 1977; Leonard et al., 1978). In these compounds, the adenine ring is laterally extended (2.4 Å) by the formal insertion of a benzene ring into the center of the purine ring system, the enzyme-binding characteristics at the terminal rings are preserved, and the potential for π interaction is increased. These dimensional probes can help set limitations on the size and flexibility of enzyme-binding sites.

An advantage of *lin*-benzoadenine nucleotides over commonly used nucleotide analogues is that the very method of synthesis (Leonard et al., 1978) precludes contamination by natural adenine nucleotides. Such contamination can cause discrepancies and confusion and has been reported and/or discussed (Kaplan & Coleman, 1978; Jebeleanu et al., 1974).

The present studies were undertaken with the intention of learning more about the specificity and restrictions of adenine nucleotide binding sites of the enzymes of oxidative phosphorylation. In this work we report the interactions of *lin*-benzo-ADP and *lin*-benzo-ATP with the mitochondrial ATP synthetase, the purified mitochondrial ATPase, and the mitochondrial adenine nucleotide carrier.

Experimental Procedure

Beef heart mitochondria (Crane et al., 1956), submitochondrial particles (Schuster et al., 1975), and mitochondrial ATPase (F_1) (Knowles & Penefsky, 1972) were prepared according to the procedures cited. Rat liver mitochondria were prepared according to the procedure of Johnson & Lardy (1967). The homogenizing medium contained, in addition, 0.1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid and 0.5 mg/mL bovine serum albumin (low fatty acid type) which were absent from the washing medium. For mitochondria and submitochondrial particles, protein was determined by the biuret reaction in the presence of 1% sodium deoxycholate, while for F_1 the method of Lowry et al. (1951) was employed. In both cases, the protein standard was crystalline bovine

† From the Institute for Enzyme Research and the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706, and the Roger Adams Laboratory, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801. *Received March 6, 1978.* This work was supported at the University of Wisconsin by Grant AM-10,334 from the National Institutes of Health and at the University of Illinois by Grant GM-05829 from the National Institutes of Health, U.S. Public Health Service.

[1] Abbreviations used: the prefix *lin* refers to the linear disposition of the pyrimidine, benzene, and imidazole rings in the "stretched-out" version of the adenine nucleus in *lin*-benzoadenine, chemical name, 8-aminoimidazo[4,5-*g*]quinazoline; *lin*-benzo-ADP, *linear*-benzoadenosine 5′-diphosphate; *lin*-benzo-ATP, *linear*-benzoadenosine 5′-triphosphate; Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Heps, *N*-2-hydroxyethylpiperazine-*N*′-2-propanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/78/0417-3686$01.00/0

serum albumin (low fatty acid type). The protein concentration of F_1 determined by the above procedure was divided by 1.18 to obtain the dry weight of the enzyme (Kagawa & Racker, 1966). Molar concentrations of F_1 were based on a molecular weight of 360 000 (Lambeth et al., 1971).

Nucleoside triphosphate hydrolysis by F_1 was usually assayed at 30 °C in a total volume of 1.0 mL containing 50 mM Heps-KOH, pH 8.0, 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM NADH, 60 μg of pyruvate kinase, 30 μg of lactate dehydrogenase, 1.9 mM phosphoenolpyruvate, and variable concentrations of nucleotides. Scopes et al. (1977) have reported that *lin*-benzoadenosine 5′-diphosphate is indeed a substrate for pyruvate kinase.

Before use, F_1 was centrifuged at room temperature to remove the bulk of the $(NH_4)_2SO_4$ and ATP included in the storage medium. The pellet was resuspended in 0.25 M sucrose, 50 mM Heps-KOH, pH 8.0, at room temperature. Reactions were initiated by the addition of approximately 2 μg of F_1 and monitored by the disappearance of NADH absorbance at 360 nm using a millimolar extinction coefficient of 4.07. When inhibition of F_1-catalyzed ATP hydrolysis by *lin*-benzoadenosine diphosphate was examined, hydrolysis was measured by the release of $^{32}P_i$ from [γ-^{32}P]ATP as described by Schuster et al. (1976). The specific radioactivity of the [γ-^{32}P]ATP was 2.3×10^6 dpm per μmol.

Incorporation of $^{32}P_i$ into organic phosphate by rat liver mitochondria was measured at 30 °C in 5 mL of a medium containing 0.23 M mannitol, 0.07 M sucrose, 0.01 M sodium succinate, 3 mM sodium Hepes, pH 7.4, 1.5 μM rotenone, 1 mM $^{32}P_i$, and 1 mg of mitochondrial protein per mL. At various times, 0.75-mL aliquots were removed and added to 0.075 mL of 60% trichloroacetic acid at 0 °C. After centrifugation at 0 °C to remove the protein, a portion of the supernatant fluid was extracted exhaustively to remove P_i by the procedure of Pullman (1967) and the remaining organic ^{32}P was measured by scintillation counting (Cerenkov radiation).

Nucleoside triphosphate synthesis by submitochondrial particles was measured as described by Schuster et al. (1977), except that the concentration of hexokinase was 170 μg/mL. The ability of the coupling enzyme hexokinase to utilize *lin*-benzo-ATP has been reported (Scopes et al., 1977; Leonard et al., 1978). The ammonium sulfate suspension of hexokinase was dialyzed at 4 °C for 12 h vs. 10 mM Bes-KOH, 0.2 mM EDTA, pH 7.0, prior to use. The specific radioactivity of the $^{32}P_i$ was approximately 2×10^6 dpm per μmol (Cerenkov radiation).

Mitochondrial oxygen consumption was measured with a Clark electrode (Yellow Springs Inst. Co.) using a Gilson oxygraph. Experiments were carried out at a protein concentration of 2.5 mg/mL, 30 °C, in 0.23 M mannitol, 0.07 M sucrose, 0.01 M sodium succinate, 5 mM sodium phosphate, 3 mM sodium Hepes, pH 7.4, and 1.5 μM rotenone.

The transport of nucleotides across the inner membrane of mitochondria was measured by a [^{14}C]ADP displacement assay. Mitochondria were loaded with radioactive nucleotide by incubating 250–300 mg of protein with 0.5 μCi of [^{14}C]-ADP (300 mCi/mmol) in 10.0 mL of 0.23 M mannitol, 0.07 M sucrose, 3 mM sodium Hepes, pH 7.4, 0 °C, for 1 h. After washing and resuspending the mitochondria with the same medium, [^{14}C]ADP-loaded mitochondria (2–2.5 mg of protein) were incubated at 23 °C in a total volume of 1 mL containing 85 mM KCl, 34 mM Tris-HCl, pH 7.4, and 0.85 mM NaEDTA for 5 min. At this time the nonradioactive nucleotides were added. After a further incubation of 2 min, atractyloside, 50 nmol, was added in a small volume of solution and mixed, and the mitochondria were centrifuged for 2 min at 12 000 rpm (8000*g*) in an Eppendorf microcentrifuge (no. 3200). A portion of the supernatant fluid was removed for determination of radioactivity by scintillation counting in 10.0 mL of Aquasol. Leakage of [^{14}C]ADP across the inner membrane was measured by an identical procedure except that nonradioactive nucleotides were not added after the 5-min incubation. Control experiments demonstrated that the presence of atractyloside during the 5-min incubation inhibited the displacement of [^{14}C]ADP by ADP by 60–80% but had no effect on the [^{14}C]ADP leakage.

Fluorescence measurements were performed with an SLM series 8000 spectrofluorometer in the ratio mode with quinine sulfate as the reference solution. Loosely bound and unbound nucleotides were removed by a centrifuge column technique (Penefsky, 1977). The 1-mL plastic tuberculin syringe was filled to the 0.7-mL calibration mark with Sephadex G-50-80 fine, previously swollen in 25% (v/v) glycerol, 50 mM Tris-acetate, pH 7.5. Upon centrifugation at 800 rpm for 2 min (Sorvall, GLC-1), the resin shrank to about the 0.5-mL calibration mark.

A sample of F_1 (up to 5 mg) was prepared as described by Penefsky (1977), applied to the column, and eluted by centrifugation. As observed by Penefsky, the elution of F_1 from the column was nearly quantitative. The ratio of absorbance at 280 nm to that at 260 nm was 1.30 ± 0.01 ($n = 5$). This value corresponds to 3 mol of adenine nucleotides bound per mol of F_1, suggesting that nucleotides were removed from the two reversible binding sites on the enzyme (Garret & Penefsky, 1975). The solution of F_1 was diluted to an appropriate volume with 25% glycerol, 50 mM Tris-acetate, pH 7.5. Nucleotides, metals, etc. were added in small volumes, and the fluorescence of *lin*-benzo-ADP was measured at 30 °C. Under these conditions F_1 freed of loosely bound nucleotides was stable for at least 15 h at room temperature; however, decreasing the glycerol to 10% or below or raising the pH to 8.0 resulted in time-dependent losses of ATPase activity. When time-dependent changes in *lin*-benzo-ADP fluorescence were monitored, the wavelengths of excitation and emission were 331 and 373 nm, respectively. The absorbance of the solutions was low enough to avoid "inner filter" effects.

Linear double-reciprocal plots and Hill plots were derived from the data using a weighted least-squares fit, the weighting factor being the reciprocal of the variance (Wilkinson, 1961). Noncompetitive substrate inhibition by P_i was analyzed by fitting the data to the following equation:

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB + B^2/K_{i1} + AB/K_{i2}} \quad (1)$$

Kinetic constants are reported according to the definitions of Cleland (1963).

The concentrations of *lin*-benzo-ADP and *lin*-benzo-ATP were measured by absorbance at 331 nm using a millimolar extinction coefficient of 9.75 (Leonard et al., 1976).

$^{32}P_i$ was obtained from New England Nuclear, [γ-^{32}P]ATP from Amersham, and [^{14}C]ADP, uniformly labeled, from Schwarz/Mann. Hexokinase was purchased from Boehringer Mannheim. Sephadex G-50-80 was obtained from Sigma.

lin-Benzo-ADP and *lin*-benzo-ATP were synthesized by the methods of Leonard et al. (1978).

Results

Interaction of lin-Benzoadenine Nucleotides with Intact Mitochondria. As an initial assessment of whether *lin*-benzo-ADP could serve as a substrate for phosphorylation in intact mitochondria, oxygen consumption studies were carried out with mitochondria from beef heart and rat liver. In neither

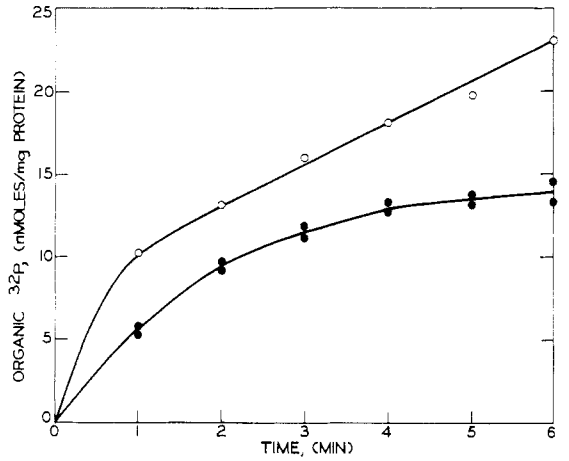

FIGURE 1: The effect of *lin*-benzo-ADP upon the incorporation of $^{32}P_i$ into organic phosphate by rat liver mitochondria. Incorporation of $^{32}P_i$ into organic phosphates was measured as described in the Experimental Procedure. The reaction was initiated by the addition of mitochondria. (●) Control, no added nucleotides; (○) 50 μM *lin*-benzo-ADP included in the medium. Under similar conditions the ADP phosphorylation was determined by O_2 consumption to be 110 nmol per min per mg of protein.

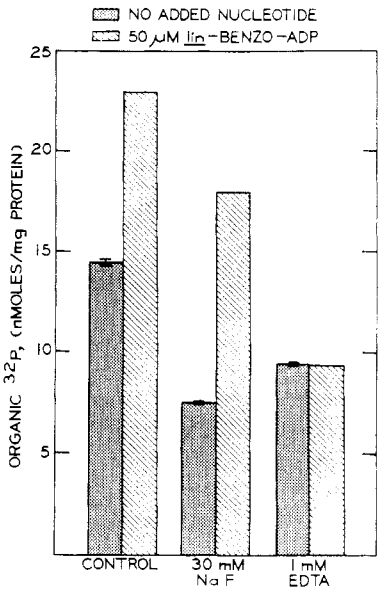

FIGURE 2: The effects of EDTA and NaF upon the enhancement by *lin*-benzo-ADP of the incorporation of $^{32}P_i$ into organic phosphates by rat liver mitochondria. Incorporation of $^{32}P_i$ into organic phosphates was measured as described in the Experimental Procedure. All reactions were initiated by the addition of mitochondria and terminated after a 5-min incubation. When measured, the standard error is indicated by error bars.

TABLE I: Displacement of Mitochondrial [^{14}C]ADP by *lin*-Benzoadenosine 5′-Diphosphate and *lin*-Benzoadenosine 5′-Triphosphate.[a]

	additions	incubation time (min)	[^{14}C]ADP displaced (%)
(I)	200 μM ADP	1	63 ± 1.0
	200 μM ADP	2	77 ± 0.9
	+200 μM *lin*-benzo-ADP	2	75 ± 1.0
	+400 μM *lin*-benzo-ADP	2	77 ± 0.7
	+800 μM *lin*-benzo-ADP	2	77 ± 1.1
	200 μM *lin*-benzo-ADP	2	1.5 ± 1.2
	400 μM *lin*-benzo-ADP	2	2.5 ± 0.9
	800 μM *lin*-benzo-ADP	2	3.8 ± 1.9
(II)	200 μM ATP	2	68 ± 1.3
	+200 μM *lin*-benzo-ATP	2	65 ± 1.3
	+400 μM *lin*-benzo-ATP	2	70 ± 1.5
	+800 μM *lin*-benzo-ATP	2	68 ± 2.8
	200 μM *lin*-benzo-ATP	2	1.2 ± 0.8
	400 μM *lin*-benzo-ATP	2	2.2 ± 1.3
	800 μM *lin*-benzo-ATP	2	2.4 ± 1.8

[a] Experiments were performed as described in the Experimental Procedure. The control value for [^{14}C]ADP leakage has been subtracted. All values are averages of duplicate determinations. Figures given are the average ± (the deviation from the average plus the standard error (n = 4) for the control value).

case did 220 μM *lin*-benzo-ADP stimulate respiration nor did it inhibit the stimulation of respiration caused by the addition of 150 μM ADP to the energized beef heart mitochondria. These data indicate that at best *lin*-benzo-ADP is a poor substrate with either the adenine nucleotide carrier or the membrane bound coupling factor. Consequently, translocation and phosphorylation of *lin*-benzo-ADP in intact mitochondria were examined.

The activity of *lin*-benzo-ADP and *lin*-benzo-ATP with the adenine nucleotide carrier of rat liver mitochondria is examined in Table I. Control experiments showed that 200 μM ADP or ATP were able to displace radioactivity in a time dependent fashion from the mitochondria loaded with [^{14}C]ADP. Concentrations of *lin*-benzo-ADP or *lin*-benzo-ATP up to 800 μM failed to inhibit the displacement of [^{14}C]ADP caused by 200 μM ADP or by 200 μM ATP. However, the *lin*-benzoadenine nucleotides were able to displace only small percentages of the [^{14}C]ADP in an apparent concentration-dependent manner.

The interaction of *lin*-benzo-ADP with energized, intact mitochondria is examined in Figures 1 and 2. In Figure 1 the incorporation of $^{32}P_i$ into endogenous nucleotides leveled off at 14 nmol/mg of protein. This value is equal, within experimental error, to the total adenine nucleotide content of rat liver mitochondria (14.3 ± 2.5 nmol/mg of protein) as reported in Altman & Katz (1976). When 50 μM *lin*-benzo-ADP was present, the initial rate of $^{32}P_i$ incorporation was enhanced and the steady-state rate, 2.5 nmol per min per mg of protein, was 2% of the ADP phosphorylation rate measured by O_2 consumption. The magnitude of incorporation with *lin*-benzo-ADP was greater than the maximum value observed in the control. This increase in the magnitude of incorporation is examined further in Figure 2. In the presence of 30 mM fluoride ion, an inhibitor of the mitochondrial adenylate kinase (Barkulis & Lehninger, 1951), the increase in the $^{32}P_i$ incorporation was not significantly affected. In the presence of 1 mM EDTA, however, the increase was abolished. In control experiments with externally added ADP, EDTA had no effect on either the ADP-stimulated respiration rate or on the P/O ratio.

Interaction of lin-Benzoadenine Nucleotides with Submitochondrial Particles. Initial studies demonstrated that *lin*-benzo-ADP was a substrate for phosphorylation by submitochondrial particles from beef heart and rat liver. The phosphorylation was (1) linear with time and extrapolated through the origin, (2) dependent upon the presence of an oxidizable substrate in the medium, and (3) nearly abolished by 200 μM 2,4-dinitrophenol.

Before attempting to compare the kinetics of phosphorylation of *lin*-benzo-ADP and ADP we sought conditions that would simplify the kinetic patterns. In previous studies (Schuster et al., 1977), the kinetics of ATP synthesis were complex and the data could not be fitted to standard equations

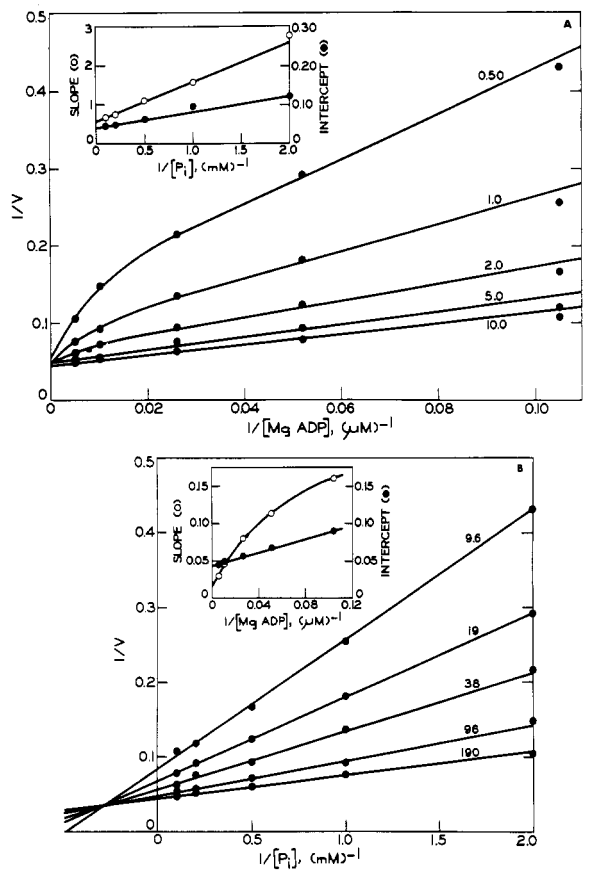

FIGURE 3: Initial velocity patterns for ATP synthesis by beef heart submitochondrial particles. ATP synthesis was measured as described in the Experimental Procedure. The velocities are expressed as nmol min^{-1} mg^{-1}. (A) ADP is the variable substrate and the fixed millimolar concentrations of P_i are indicated in the Figure. Insert: replot of slopes and intercepts from the linear portions of the data. (B) P_i is the variable substrate and the fixed micromolar concentrations of ADP are indicated in the figure. Insert: replot of slopes and intercepts from the data.

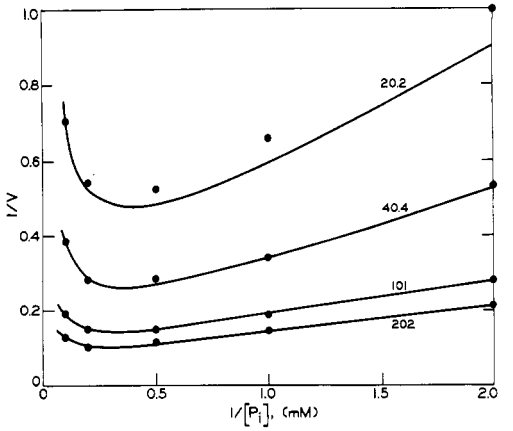

FIGURE 4: Substrate inhibition by P_i of *lin*-benzo-ADP phosphorylation by beef heart submitochondrial particles. Phosphorylation of *lin*-benzo-ADP was measured as described in the Experimental Procedure. P_i is the variable substrate, and the fixed micromolar concentrations of *lin*-benzo-ADP are indicated in the figure. Data were fitted to eq 2 with ADP as A and P_i as B. The velocities are expressed as nmol min^{-1} mg^{-1}.

TABLE II: Kinetic Constants for Phosphorylation of ADP and *lin*-Benzo-ADP by Beef Heart Submitochondrial Particles.[a]

	constant	value
(I)	*lin*-benzo-ADP	
	V_m	24 ± 4 nmol/(min·mg)
	apparent $K_{l\text{-}b\text{-}ADP}$[b]	90 ± 40 μM
	apparent $K_{i\text{-}l\text{-}b\text{-}ADP}$[c]	120 ± 40 μM
	apparent K_{P_i}	1.1 ± 0.4 mM
	K_{i1}	19 ± 2 mM
	K_{i2}	23 ± 18 mM
(II)	ADP	
	V_m	23.2 ± 0.5 nmol/(min·mg)
	K_{ADP}	10.5 ± 0.5 μM
	K_{iADP}	21 ± 0.5 μM
	K_{P_i}	1.4 ± 0.2 mM

[a] Nucleoside triphosphate synthesis was measured as described in the Experimental Procedure. [b] Apparent K for *lin*-benzo-ADP. [c] Apparent K_i for *lin*-benzo-ADP.

for enzyme-catalyzed reactions. During the course of this work, it was discovered that, if the coupling enzyme, hexokinase, was dialyzed prior to use in order to remove the $(NH_4)_2SO_4$, the kinetic patterns were greatly simplified. In Figure 3A, the kinetics of ATP synthesis by beef heart submitochondrial particles with ADP as the variable substrate are examined. At low fixed concentrations of P_i, the double-reciprocal plots exhibited negative cooperativity with respect to ADP. The Hill coefficients varied from 0.69 at 0.5 mM P_i to 0.93 at 10 mM P_i. The linear portions of the data were fitted to the following equation:

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (2)$$

that predicts the initial velocities of a sequential enzyme-catalyzed reaction with two substrates. The slope and intercept replots are linear (see insert to Figure 3A) indicating a good fit to the equation. With P_i as the variable substrate (Figure 3B), no curvature was observed in the double-reciprocal plots. The intercept replot was linear while the slope replot was concave downward (see insert to Figure 3B) with a Hill coefficient of 0.68. The kinetic constants of eq 2 were obtained from these data and are presented in Table II. In agreement with a previous report (Schuster et al., 1977), when the hexokinase was not dialyzed, the double-reciprocal plots of ATP synthesis with ADP as the variable substrate failed to exhibit cooperativity (Hill coefficient = 0.98 at 0.5 mM P_i).

When *lin*-benzo-ADP was used as a substrate for phosphorylation, the kinetic patterns differed significantly from those in Figure 3. High concentrations of P_i caused substrate inhibition at all fixed concentrations of *lin*-benzo-ADP (Figure 4). With *lin*-benzo-ADP as the variable substrate the double-reciprocal plots were linear with Hill coefficients ranging from 0.99 to 1.01 (data not shown). P_i affected both the slope and intercept replots of the latter data, demonstrating that the substrate inhibition was noncompetitive. Consequently, the data were fitted to eq 1, and the kinetic constants are presented in Table II. Note that, while the V_m for ADP and *lin*-benzo-ADP are equal, within experimental error, the apparent K_m for *lin*-benzo-ADP is about nine times the K_m for ADP.

Interaction of lin-Benzoadenine Nucleotides with the Purified ATPase from Beef Heart Mitochondria. The hydrolysis of *lin*-benzo-ATP by F_1 was demonstrated by means of a coupled enzyme assay (pyruvate kinase and lactate dehydrogenase). The loss of NADH absorbance was completely dependent on the presence of F_1. The kinetics of hydrolysis and the effect of HCO_3^-, an activating anion, were very similar to the results from previous studies with ATP as the substrate (Ebel & Lardy, 1975). In the absence of HCO_3^-, the double-reciprocal plot for hydrolysis of *lin*-benzo-ATP displayed negative cooperativity with a Hill coefficient nearly equal to

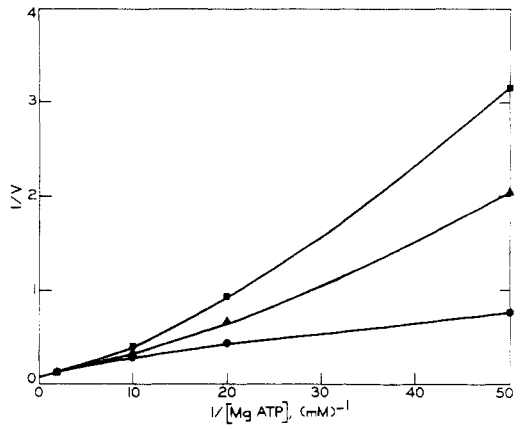

FIGURE 5: The effect of *lin*-benzo-ADP upon ATP hydrolysis catalyzed by the purified mitochondrial ATPase. Hydrolysis of ATP was measured by the release of $^{32}P_i$ from [γ-^{32}P]ATP as described in the Experimental Procedure. The velocities are expressed as μmol min^{-1} mg^{-1}. (●) Control; (▲) 10 μM ADP; (■) 10 μM *lin*-benzo-ADP.

TABLE III: F_1-Catalyzed Nucleoside Triphosphate Hydrolysis; Kinetic Parameters for Nucleotides.[a]

	-HCO_3^-		10 mM HCO_3^-	
substrate	V_m (μmol/(min·mg))	Hill coeff	V_m (μmol/(min·mg))	K_m (μM)
ATP	ca. 50	0.67	94 ± 2	186 ± 7
lin-benzo-ATP	ca. 40	0.63	56 ± 1	187 ± 6

[a] Nucleoside triphosphate hydrolysis was measured as described in the Experimental Procedure.

the value for ATP hydrolysis. As with ATP, the negative cooperativity was abolished when 10 mM HCO_3^- was included in the reaction medium—the Hill coefficients increased to 1.00 for ATP and 0.99 for *lin*-benzo-ATP. The kinetic constants for these data are presented in Table III.

ADP is known to be an inhibitor of ATP hydrolysis catalyzed by the purified mitochondrial ATPase (Hammes & Hilborn, 1971; Schuster et al., 1976). Consequently, the ability of *lin*-benzo-ADP to inhibit ATP hydrolysis was investigated. In Figure 5, as observed previously (Schuster et al., 1976), ADP is a competitive inhibitor of ATP hydrolysis with complex kinetics of inhibition. Inhibition by *lin*-benzo-ADP is also competitive and complex. Assuming that the inhibition is linear competitive at high ATP concentrations, the inhibition constants (K_i) were estimated to be 27 μM for ADP and 16 μM for *lin*-benzo-ADP. The K_i (ADP) estimated in this manner was approximately equal to the value (30 μM) previously reported by Hammes & Hilborn (1971).

Since *lin*-benzoadenine nucleotides are fluorescent (Scopes et al., 1977; VanDerLijn et al., 1978), experiments were designed to test whether F_1 influences the fluorescence emission of *lin*-benzo-ADP. As seen in Figure 6, the fluorescence spectrum of 1 μM *lin*-benzo-ADP was extensively quenched (85%) by 5 μM F_1. Two kinetically distinct phases of quenching were observed. An initial rapid phase representing 80% of the total quenching was complete within 10–15 min. In addition, a very slow, first-order quenching phase with a half-life of 25 min was observed. ADP, 50 μM, was able to reverse 75% of the total quenching by F_1 within 10–15 min. Following this reversal, however, a slow phase of quenching was reinitiated (data not shown).

When Mg^{2+} was omitted from the medium, a single phase

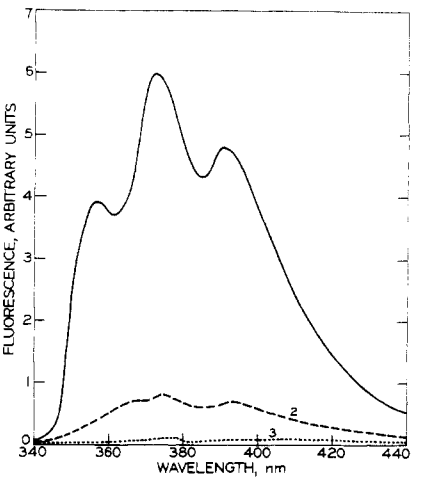

FIGURE 6: The effect of the purified mitochondrial ATPase (F_1) upon the fluorescence of *lin*-benzo-ADP in the presence of Mg^{2+}. Fluorescence spectra were determined as described in the Experimental Procedure. The concentration of $MgCl_2$ was 5 mM; (1) 1 μM *lin*-benzo-ADP; (2) 1 μM *lin*-benzo-ADP + 5 μM F_1; (3) 5 μM F_1.

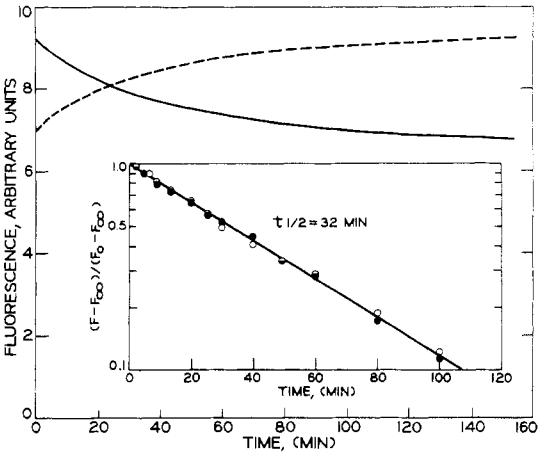

FIGURE 7: Quenching of *lin*-benzo-ADP fluorescence by F_1 and reversal of quenching by ADP in the absence of Mg^{2+}. The fluorescence signal was monitored as described in the Experimental Procedure. The excitation and emission wavelengths were 331 and 373 nm, respectively. The concentrations of F_1 and *lin*-benzo-ADP were 5 μM and 1 μM, respectively. (—) Quenching was initiated by the addition of F_1; (- - -) the reversal of quenching was initiated by the addition of 50 μM ADP after F_1 and *lin*-benzo-ADP had been incubated for 180 min. Insert: the above data were fitted to an equation for a first-order decay or appearance of fluorescence. (●) Fluorescence quenching by F_1; (○) reversal of quenching by 50 μM ADP.

of quenching with a half-life of 32 min was observed (Figure 7). The extent of quenching, 25%, was much lower than when Mg^{2+} was present. Unlike the situation when Mg^{2+} is present, 50 μM ADP completely reversed this fluorescence quenching by F_1. The kinetics of quenching by F_1 and reversal by ADP were identical (see insert to Figure 7).

Discussion

Certain aspects of these studies bear striking similarities to the results of previous studies with adenine nucleotide analogues. Those aspects are (a) the stringent specificity of the adenine nucleotide carrier for the heteroaromatic component of the nucleotide (cf. Schlimme et al., 1977, and references therein), and (b) the relative lack of specificity of the mitochondrial ATP-synthetase, the purified mitochondrial ATPase, and the mitochondrial nucleoside diphosphokinase for the heteroaromatic portion of the nucleotide (Löw et al., 1963;

Hohnandel & Cooper, 1972; Wagenvoord et al., 1977; Kozlov & Skulachev, 1977; Bârzu et al., 1976; Jebeleanu et al., 1974). The present data, therefore, confirm and extend the specificities of the enzymes described above as predicted from past studies.

In contrast to the present results with *lin*-benzoadenine nucleotides, Bârzu et al. (1976) and Schlimme et al. (1973) concluded that etheno-ADP and the N^1-oxide of ADP are not substrates for phosphorylation by the mitochondrial ATP-synthetase. Apparently these two derivatives of ADP are exceptions to the lack of specificity generally observed, although phosphorylation of ϵ-ADP has been observed in chloroplasts (Schahak et al., 1973).

The experiments with intact mitochondria deserve further comment. Since *lin*-benzo-ADP displaced small amounts of [^{14}C]ADP from labeled mitochondria and since *lin*-benzo-ADP increased the incorporation by mitochondria of $^{32}P_i$ into the organic phosphates, it might appear reasonable to conclude that the ADP analogue is a substrate of the adenine nucleotide carrier. However, this conclusion is not supported by the data in Figure 2. EDTA abolished the enhanced incorporation of $^{32}P_i$ into organic phosphates but had no effect on the phosphorylation of added ADP. From this it follows that the incorporation is not catalyzed directly by the membrane bound ATP synthetase. Since *lin*-benzo-ADP is known to be phosphorylated by rat liver submitochondrial particles (see Results), it appeared unlikely that the nucleotide analogue was transported across the inner membrane at an appreciable rate.

The matrix space of the mitochondrion is inaccessible to EDTA (Harris & Van Dam, 1968; Puskin & Gunter, 1973). This indicates that the enzyme(s) responsible for the phosphoryl transfer to *lin*-benzo ADP must be located in or accessible at one of the following: the outer membrane, the intermembrane space, or the outer side of the inner membrane. In view of our current knowledge of mitochondrial enzymes and their locations (Altman & Katz, 1976), two likely sites at which EDTA is exerting its inhibition are adenylate kinase and nucleoside diphosphokinase, both of which depend upon divalent cations for activity (Noda, 1973; Parks & Agarwal, 1973). As shown in Figure 2, 30 mM fluoride ion, an inhibitor of the mitochondrial adenylate kinase (Barkulis & Lehninger, 1951), had little or no effect upon the incorporation of $^{32}P_i$ enhanced by *lin*-benzo-ADP. The participation of adenylate kinase in the phosphate transfer, therefore, is rendered unlikely. In summary, the data suggest that small amounts of ^{32}P-labeled endogenous nucleotides cross the inner membrane and catalyze net phosphorylation of *lin*-benzo-ADP through the action of nucleoside diphosphokinase in the intermembrane space.

The mechanism for the displacement of small amounts of [^{14}C]ADP from labeled mitochondria by *lin*-benzo-ADP is not known with certainty. The above findings indicate that this displacement does not represent transport of the nucleotide analogue across the inner membrane in exchange for ADP. Relevant to this discussion may be the finding that UDP, GDP, and CDP bind to mitochondrial membranes and/or enzymes in an atractyloside insensitive manner (Duée & Vignais, 1969). If one assumes that *lin*-benzo-ADP can bind to such sites and that these sites are normally occupied by ADP, then the displacement of radioactivity is reasonable.

During the course of this work it was discovered that the removal of $(NH_4)_2SO_4$ (carried over with the coupling enzyme hexokinase) from the reaction medium simplified the kinetics of ATP synthesis by beef heart submitochondrial particles. Using this finding the Michaelis constants for P_i and ADP were determined to be 1.4 mM and 10.5 μM, respectively. This K_m for ADP is approximately equal to the value, 12 μM, obtained with rat liver submitochondrial particles (Hohnandel & Cooper, 1972), and significantly lower than a value (300 μM) previously reported for beef heart submitochondrial particles (Bygrave & Lehninger, 1967). The K_m for P_i measured in this report is about one-sixth of a previously determined value (Bygrave & Lehninger, 1967). All of the above-mentioned K_m values from the literature were determined at a single fixed concentration of the second substrate.

In addition to simplifying the kinetics of ATP synthesis, the removal of $(NH_4)_2SO_4$ from the reaction medium revealed negative cooperativity with respect to ADP in the direction of ATP synthesis. Previously cooperativity had been observed only in the direction of ATP hydrolysis (Ebel & Lardy, 1975; Pedersen, 1976). The negative cooperativity was maximal at 0.5 mM P_i (since the Hill coefficients for the slopes vs. 1/MgADP and for ATP synthesis at 0.5 mM P_i were equal) and was nearly absent at 10 mM P_i. Although more information is needed before a mechanism can be formulated, the negative cooperativity is significant at K_m levels of P_i (Hill coefficient = 0.72 at 1 mM P_i) and thus could play a physiological role in the regulation of ATP synthesis.

When the kinetics of phosphorylation of *lin*-benzo-ADP were examined, the noncompetitive substrate inhibition by high concentrations of P_i was an unexpected finding. Since no inhibition was observed when ADP was the substrate (Figure 4), the data suggest but do not prove that P_i competes with the nucleotide diphosphate for a site (not necessarily the catalytic site) and that ADP has a higher affinity for that site than does *lin*-benzo-ADP. The inhibition is actually more complex than this, since the above explanation would predict competitive substrate inhibition. In the case of a purified enzyme with a single binding site for the substrate, substrate inhibition can usually be interpreted as the formation of a dead-end complex at the catalytic site (Cleland, 1970). In the case of submitochondrial particles, however, many different binding sites and/or modes of inhibition may exist due to the complexity of the system. Nevertheless, it is interesting that the lateral extension of the adenine ring by 2.4 Å "permits" high concentrations of P_i to inhibit nucleoside triphosphate synthesis, and such a phenomenon deserves further study. Also of interest from the kinetics of phosphorylation is the finding that the K_m for P_i is not significantly altered with *lin*-benzo-ADP as substrate (Table II). Apparently the lateral extension of the heterocycle by 2.4 Å does not result in a large enough displacement of the diphosphate moiety to affect P_i binding.

When compared as substrates for hydrolysis by the purified ATPase, *lin*-benzo-ATP and ATP proved to be quite similar. Both compounds displayed negative cooperativity and activation by HCO_3^-. In addition, the Michaelis constants for ATP and *lin*-benzo-ATP determined in the presence of 10 mM HCO_3^- were equal. It is surprising that *lin*-benzo-ADP is more potent ($K_i \cong 16$ μM) than ADP as an inhibitor of ATP hydrolysis.

The fluorescence studies provide independent evidence for the binding of *lin*-benzo-ADP to the purified ATPase. The rapid phase of quenching is interpreted to represent binding of the magnesium complex of *lin*-benzo-ADP since that phase is not observed when Mg^{2+} is omitted from the medium and it is known that Mg^{2+} does not quench the fluorescence of *lin*-benzo-ADP (VanDerLijn et al., 1978). The mechanism of the slow phase of quenching is not known in detail but it also represents binding since in the absence of Mg^{2+} this quenching is completely reversed by 50 μM ADP. An interesting possibility is that the slow phase of quenching represents exchange

of *lin*-benzo-ADP with the tightly bound nucleotides on F_1.

In conclusion, the lateral extension of the adenine ring by 2.4 Å modifies the kinetics of ATP synthesis by submitochondrial particles but has little effect upon kinetics of ATP hydrolysis by the purified mitochondrial ATPase. Overall, *lin*-benzo-ADP and *lin*-benzo-ATP substituted well for adenine nucleotides as substrates for the enzymes of oxidative phosphorylation. Although the possibility of a low rate of transport across the inner membrane has not been ruled out, the data demonstrate that *lin*-benzo-ADP exhibits little, if any, activity as a substrate for the adenine nucleotide carrier.

Acknowledgment

The technical assistance of Marion J. Wagner with portions of this work is appreciated. The FORTRAN program for eq 1 was supplied by Professor W. W. Cleland.

References

Altman, P. L., & Katz, D. D. (1976) *Cell Biology,* Federation of American Societies for Experimental Biology, p 143, Bethesda, Md.

Barkulis, S. S., & Lehninger, A. L. (1951) *J. Biol. Chem. 190,* 339.

Bârzu, O., Kiss, L., Bojan, O., Niac, G., & Mantsch, H. H. (1976) *Biochem. Biophys. Res. Commun. 73,* 894.

Bygrave, F. L., & Lehninger, A. L. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57,* 1409.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67,* 104; 173.

Cleland, W. W. (1970) *Enzymes, 3rd Ed. 2,* 1.

Crane, F. L., Glenn, J. L., & Green, D. E. (1956) *Biochim. Biophys. Acta 22,* 475.

Duée, E. D., & Vignais, P. V. (1969) *J. Biol. Chem. 244,* 3920.

Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250,* 191.

Garret, N. E., & Penefsky, H. S. (1975) *J. Biol. Chem. 250,* 6640.

Hammes, G. G., & Hilborn, D. A. (1971) *Biochim. Biophys. Acta 233,* 580.

Harris, E. J., & Van Dam, K. (1968) *Biochem. J. 106,* 759.

Hohnandel, D. C., & Cooper, C. (1972) *Biochemistry 11,* 1138.

Jebeleanu, G., Ty, N. G., Mantsch, H. H., Bârzu, O., Niac, G., & Abrudan, I. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 4630.

Johnson, D., & Lardy, H. A. (1967) *Methods Enzymol. 10,* 94.

Kagawa, Y., & Racker, E. (1966) *J. Biol. Chem. 241,* 2461, 2467.

Kaplan, R. S., & Coleman, P. S. (1978) *Biochim. Biophys. Acta 501,* 269.

Knowles, A. F., & Penefsky, H. S. (1972) *J. Biol. Chem. 247,* 6617.

Kozlov, I. A., & Skulachev, V. P. (1977) *Biochim. Biophys. Acta 463,* 29.

Lambeth, D. D., Lardy, H. A., Senior, A. E., & Brooks, J. C. (1971) *FEBS Lett. 17,* 330.

Leonard, N. J., Sprecker, M. A., & Morrice, A. G. (1976) *J. Am. Chem. Soc. 98,* 3987.

Leonard, N. J., Scopes, D. I. C., VanDerLijn, P., & Barrio, J. R. (1978) *Biochemistry 17* (preceding paper in this issue).

Low, H., Vallin, I., & Alm, B. (1963) in *Energy Linked Functions of Mitochondria* (Chance, B., Ed.) p 5, Academic Press, New York, N.Y.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J/ Biol. Chem. 193,* 265.

Noda, L. (1973) *Enzymes, 3rd Ed. 8,* 279.

Parks, R. E., Jr., & Agarwal, R. P. (1973) *Enzymes, 3rd Ed. 8,* 307.

Pedersen, P. L. (1975) *Bioenergetics 6,* 243.

Pedersen, P. L. (1976) *J. Biol. Chem. 251,* 934.

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

Pullman, M. E. (1967) *Methods Enzymol. 10,* 57.

Puskin, J. S., & Gunter, T. E. (1973) *Biochem. Biophys. Res. Commun. 51,* 797.

Schlimme, E., Lamprecht, W., Eckstein, F., & Goody, R. S. (1973) *Eur. J. Biochem. 40,* 485.

Schlimme, E., Boos, K. S., Bojanovski, D., & Lustorff, J. (1977) *Angew. Chem., Int. Ed. Engl. 16,* 695.

Schuster, S. M., Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250,* 7848.

Schuster, S. M., Gertschen, R. J., & Lardy, H. A. (1976) *J. Biol. Chem. 251,* 6705.

Schuster, S. M., Reinhart, G. D., & Lardy, H. A. (1977) *J. Biol. Chem. 252,* 427.

Scopes, D. I. C., Barrio, J. R., & Leonard, N. J. (1977) *Science 195,* 296.

Shahak, Y., Chipman, D. M., & Shavit, N. (1973) *FEBS Lett. 33,* 293.

VanDerLijn, P., Barrio, J. R., & Leonard, N. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* (in press).

Wagenvoord, R. J., Van Der Kraan, I., & Kemp, A. (1977) *Biochim. Biophys. Acta 460,* 17.

Wilkinson, G. N. (1961) *Biochem. J. 71,* 324.