Equilibrium Binding of Nucleotides to Beef Heart Mitochondrial Adenosine Triphosphatase†

David A. Hilborn‡ and Gordon G. Hammes*

ABSTRACT: Equilibrium binding studies of various nucleotides to the solubilized ATPase from beef heart mitochondria have been carried out utilizing ultrafiltration, gel filtration, fluorometric, and spectrophotometric techniques. Direct binding studies with ADP at pH 8.0 in the presence of divalent magnesium indicate that two distinctly different binding sites per enzyme molecule exist, a "tight" site with a dissociation constant of 0.28 μM and a "loose" site, which is also the catalytic site, with a dissociation constant of 47 μM. In the absence of a divalent metal ion the binding of this diphosphate to the "tight" site is weaker while its binding to the "loose" site remains essentially unchanged. Of the nucleotides studied at pH 8.0, the "tight" site interacted most strongly with ADP and its fluorescent analog, 3-β-D-ribofuranosylimidazo[2,1-*i*]-purine 5′-diphosphate. Ultraviolet difference spectroscopy was used to determine the dissociation constant (42 μM) for the binding of 6-mercapto-9-β-D-ribofurnosylpurine 5′-diphosphate to the "tight" site. Quantitative information about the binding specificity for the interaction of the solubilized enzyme with other nucleotides (AMP, ATP, IDP, ITP, UDP, and UTP) is presented, as well as evidence suggesting that the "tight" site may be missing or the binding considerably weakened when the ATPase is attached to its native mitochondrial membrane.

The ATPase from beef heart mitochondria catalyzes an Mg^{2+}-dependent dinitrophenol-stimulated hydrolysis of ATP to ADP and inorganic phosphate. Since its initial purification by Pullman *et al.* (1960), its chemical and physical properties as well as its functional role in oxidative phosphorylation have been extensively studied (*cf.* Horstman and Racker, 1970, and MacLennan and Tzagoloff, 1968, and references therein). Although various procedures for the purification of this ATPase have been reported (Horstman and Racker, 1970; Senior and Brooks, 1970; Datta and Penefsky, 1970), which yield proteins with slightly differing physical properties, the central importance of this enzyme in oxidative phosphorylation is generally acknowledged.

Apart from its role in oxidative phosphorylation, this ATPase also can be utilized for studying the interaction of an enzyme with its native membrane. Mitochondrial membrane fragments devoid of the ATPase can be readily prepared, and the membrane-bound ATPase can be reconstituted by simply mixing the solubilized ATPase with these membrane fragments (Penefsky *et al.*, 1960). The membrane-bound ATPase retains many of the properties it had in intact mitochondria so that the solubilized and membrane-bound enzymes can be readily compared. The kinetic properties of the two forms of the ATPase have been shown to have significant differences (Hammes and Hilborn, 1971).

In this study equilibrium binding measurements of a series of nucleotides to the solubilized ATPase were carried out using a variety of experimental approaches. The number, types, and nucleotide specificities of the binding sites on the enzyme have been determined as well as the dissociation constants for several of the nucleotides. In addition, measurements of the binding of 3-β-D-ribofuranosylimidazo[2,1-*i*]-purine diphosphate (ϵADP,[1] an ADP fluorescent analog; Secrist *et al.*, 1972) and ADP to the membrane-bound ATPase suggest that the number and/or the binding affinity of nucleotide binding sites depends on whether the enzyme is solubilized or attached to its native membrane.

Experimental Section

Materials. Commercially available nucleotides (ATP, ADP, AMP, ITP, IDP, UDP, UTP, and SHMP) were purchased from either Sigma Chemical Co. or P-L Biochemicals, Inc. The Trizma Base was obtained from Sigma Chemical Co.; Sephadex was purchased from Pharmacia Fine Chemicals, Inc. The Cellex-D (DEAE-cellulose) was purchased from Bio-Rad Laboratories. The radioactive nucleotides, [^{3}H]ADP (5–15 Ci/mmol) and [^{14}C]ADP (30–40 mCi/mmol), were obtained from New England Nuclear and purified using paper chromatography with the solvent system *n*-butyl alcohol–acetone–acetic acid–5% ammonium hydroxide–water (35:25:15:15:10, v/v). All other chemicals used were the best available commercial grades.

The preparations of the ATPase (Horstman and Racker, 1970) and the ASU particles (Racker and Horstman, 1967) from beef heart mitochondria have been described elsewhere. Beef heart mitochondria were generously provided by Dr. E. Racker. The purified ATPase sometimes produced a turbid solution when dissolved at high concentrations. To remove the turbidity, the entire ATPase preparation in the form of the ammonium sulfate precipitate was centrifuged at 4° for 10 min at 18,000*g*, and the supernatant discarded. The precipitate was warmed to room temperature and dissolved in a pH 7.4 buffer containing 0.25 M sucrose, 10 mM Tris-sulfate, 2 mM EDTA, and 30 mM ATP to a final protein concentration of approximately 8 mg/ml. After centrifugation at 25° for 20 min at 37,000*g*, the supernatant was carefully removed and

† From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received October 16, 1972.* This work was supported by a grant from the National Institutes of Health (GM 13292).

‡ National Institutes of Health Predoctoral Trainee (GM 0834); present address: The Salk Institute, San Diego, Calif. 92112.

[1] Abbreviations used are: ϵADP, 3-β-D-ribofuranosylimidazo[2,1-*i*]-purine 5′-diphosphate; SHMP, SHDP, and SHTP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-monophosphate, 5′-diphosphate, and 5′-triphosphate, respectively; ASU particles, beef heart mitochondrial membrane fractions.

the small white residue discarded. The final step in the ATPase preparation, an ammonium sulfate precipitation, was then carried out with the supernatant, and the precipitate was stored at 4°.

The ammonium sulfate suspension of the purified ATPase was found to contain small amounts of ATP. This ATP was removed from the enzyme by the following procedure. Typically 1–2 ml of the ammonium sulfate precipitated ATPase was centrifugated at 4° for 10 min at 18,000*g*, and the supernatant was discarded. The precipitate was allowed to warm to room temperature and then was dissolved in a small volume of a pH 8.0 buffer containing 0.025 M sucrose, 10 mM Tris-sulfate, and 2 mM EDTA. This solution was eluted through a Sephadex G-25 (medium) column (1.13 cm^2 × 54 cm) equilibrated with the same buffer. The effluent was monitored by passing it through a microflow cell (Helma Cell, Inc., Type 178-QS, 10 mm path length) in a Cary 14 recording spectrophotometer and collecting the protein as measured by the absorbance at 280 nm. After elution of the protein the monitoring wavelength was changed to 259 nm to observe the elution of the ATP, and to ensure that good separation of the protein and nucleotide had occurred. An equal volume of saturated ammonium sulfate, pH 7.0, immediately was added to the protein fraction to precipitate the ATPase; the precipitate was stored at 4° and was used within 1 day. The specific activity of the ATPase was unchanged by this procedure.

To equilibrate the ATPase with buffers used in these experiments the ammonium sulfate precipitate of the enzyme was centrifuged at 4° for 10 min at 18,000*g*, and the supernatant was discarded. After allowing the precipitate to warm to room temperature it was dissolved in a minimum volume of the desired buffer. This solution was placed on a Sephadex G-25 column (0.28 cm^2 × 13.5 cm) which had been equilibrated with the same buffer. The protein was collected in 1 ml or less immediately following the void volume (determined using Blue Dextran 2000). Experiments were performed as quickly as possible following this equilibration because of the instability of the ATPase in the absence of added ATP.

Activity assays and steady-state velocity measurements were carried out using the pH-Stat technique (Hammes and Hilborn, 1971). The specific activity of the enzyme was 140–150 μmol/(mg min).

Protein concentrations were determined using the method of Lowry *et al.* (1951) with human serum albumin as the standard. This result was divided by 1.18 to obtain the dry weight of the enzyme (Kagawa and Racker, 1966). Molar concentrations were based on a molecular weight of 285,000 (Forrest and Edelstein, 1970; Penefsky and Warner, 1965).

Reconstituted (membrane-bound) ATPase was prepared and its concentration determined as previously described (Hammes and Hilborn, 1971). Concentrations of reconstituted ATPase are given in terms of moles of membrane-bound ATPase per liter. The activity of adenylate kinase impurity in reconstituted particles and in ASU particles was estimated by checking for radioactively labeled ATP and AMP after incubating [^{3}H]ADP with the membrane preparations. The various nucleotides were separated from one another with thin layer chromatography as described below, and the nucleotide spots were cut out and analyzed for radioactivity.

Thin Layer Chromatography. Thin layer chromatography was used to analyze the various nucleotides in this study. Commercially available precoated thin layer plates (either Eastman Chromatogram Sheet No. 6064 cellulose or microcrystalline cellulose from Schwarz/Mann) were used with a solvent system consisting of 0.15 M citric acid (pH 4.0)–95% ethanol–*n*-butyl alcohol (6:10:1, v/v). A running time of approximately 5 hr was sufficient for separation of the mono-, di-, and triphosphates.

Preparation of SHTP, SHDP, and εADP. The SHTP was prepared from SHMP by method 2 of Murphy *et al.* (1970) except that the product was chromatographed on a DEAE-cellulose column (3.14 cm^2 × 32 cm) with a linear gradient of 0.01–0.5 M ammonium acetate, pH 6.8, in 2 l. at 4°.

The SHDP was prepared by the enzymatic hydrolysis of SHTP. Membrane-bound ATPase (300 μg) was added to 40 ml of a pH 7.0 solution containing 5 mM SHTP and 5.5 mM $MgCl_2$. This reaction mixture was stirred for 20 min at room temperature and then centrifuged at 25° for 10 min at 18,000*g* to remove the insoluble membrane-bound ATPase. The supernatant was then removed and ammonium acetate was added to bring its concentration to 0.15 M (pH 6.9). After bringing the temperature to 4° this mixture was put onto a DEAE-cellulose column (3.14 cm^2 × 32 cm) equilibrated with 0.15 M ammonium acetate, pH 6.8, and then eluted with a linear gradient of 0.15–0.40 M ammonium acetate in 2 l. at 4°. Although some overlap of the SHDP and SHTP occurred in the elution, the column fractions were separated sufficiently so that a yield of about 50% SHDP was obtained. After lyophilization, the product was checked for purity with thin layer chromatography. Only trace impurities were detectable. The concentrations of SHDP and SHTP solutions were determined spectrophotometrically at 322 nm using an extinction coefficient of 23,100 cm^{-1} M^{-1} at pH 4.6 (Hampton and Maguire, 1961).

The εADP was prepared analogously to the preparation of 3-β-D-ribofuranosylimidazo[2,1-*i*]purine hydrochloride (Barrio *et al.*, 1972), except the product was purified on a DEAE-cellulose column as described for SHTP. (An alternative synthetic procedure has been recently given by Secrist *et al.* (1972).) The εADP was checked for purity with thin layer chromatography as described above. No ADP and only trace impurities of εAMP could be detected. Concentrations of εADP solutions were determined spectrophotometrically at 275 nm using an extinction coefficient of 5.6 × 10^3 cm^{-1} M^{-1} at pH 7.0 (Secrist *et al.*, 1972).

Binding Measurements. Direct binding of several nucleotides to the enzyme was studied using the gel filtration method of Hummel and Dreyer (1962). A thermostated (25°) Sephadex G-25 (medium) column was equilibrated with buffer (0.1 M NaCl, 50 mM Tris-Cl, 2.0 mM Mg^{2+}, or 1.0 mM EDTA, pH 8.0) containing the desired concentration of nucleotide. For high concentrations of nucleotide a 1.13 cm^2 × 54 cm column was used while at low concentrations of nucleotide a larger column (3.14 cm^2 × 48 cm) was used to ensure the presence of a sufficient excess of nucleotide over enzyme throughout the column. After a known amount of the ATPase (4–6 mg) in a known volume of buffer (usually 0.5 ml) without nucleotide was put onto the column, the concentration of nucleotide in the effluent was measured at the wavelength of maximum absorption of the nucleotide using a Cary 14 recording spectrophotometer and the microflow cell previously described. Either a 0–0.1 or 0–1.0 slide wire was used. The area of the trough following the protein peak was corrected for the volume of enzyme solution put on the column which did not contain nucleotide. From this corrected area and the effluent flow rate, the amount of nucleotide bound to the enzyme was calculated as well as the ratio of the moles of nucleotide bound per molecule of enzyme. At very low concentrations of ADP, where the absorbance of the nucleotide was

too small to be monitored spectrophotometrically, radioactive ADP was utilized. In these cases [^{14}C]ADP was added to the equilibrating buffer and fractions of the effluent were collected after they passed through the flow cell. These fractions were assayed for radioactivity in Bray's solution (Bray, 1960) using liquid scintillation spectrometry. The peak in radioactivity accompanying the protein and the trough immediately following it were used to measure the moles of ligand bound to the enzyme. When a comparison was possible, spectrophotometric and radioactive results were in good agreement.

An ultrafiltration method (Paulus, 1969) was used to measure the binding of ADP to both the solubilized ATPase and the membrane-bound ATPase. The ultrafiltration cell was purchased from Metalloglass, Inc., Boston, Mass. For experiments with the solubilized enzyme, Diaflo UM-10 membranes were found to be satisfactory as only a small amount of ADP was retained by them, usually corresponding to 1–2 μl of the total amount added. The protein–ligand solutions were prepared by mixing 0.5 ml of an ATPase solution (typically 20–50 μg/ml in the appropriate buffer) with 0.5 ml of [^{3}H]ADP in buffer and then pipetting 0.25-ml aliquots of this mixture into the channels of the ultrafiltration cell. Blanks for each experiment consisted of adding 0.5 ml of buffer to 0.5 ml of [^{3}H]ADP or [^{14}C]ADP in buffer and filtering 0.25 ml of these solutions simultaneously with the protein solutions. A pressure of 40 psi nitrogen was applied for about 20 min until all the solutions had passed through the membranes, after which the undersides of the membranes were rinsed with ethylene glycol (Paulus, 1969). The membranes were assayed for radioactivity in Bray's solution (Bray, 1960) by liquid scintillation spectrometry.

The binding of ADP to the membrane-bound ATPase was studied in a similar manner, except that in these experiments the blank consisted of the same amount of ASU particles as reconstituted particles (membrane-bound ATPase) in the sample channels. Millipore filters type VMWP were used in these experiments because of the long filtering times required for UM-10 membranes with the membranous protein. However, a filtering time of about 3 hr still was required. All experiments were run at least in duplicate.

Fluorescence Measurements. Fluorescence measurements involving ϵADP were made with a Perkin-Elmer Model MPF-3 fluorescence spectrometer. All fluorescence titrations were carried out with a three- to fourfold scale expansion using the zero suppression adjustment to maintain readings on scale. The fluorescent intensity was observed at 415 nm using an excitation wavelength of 320 nm to keep the fluorescence of the protein at a minimum. The optical densities of all solutions used for titrations with the solubilized ATPase and ϵADP were low enough at 320 and 415 nm to avoid interfilter effects. A typical titration with the solubilized ATPase was performed as follows. Various amounts of solubilized ATPase were added to a fluorescence cell using a 25-μl Hamilton syringe. Simultaneously the same amount of the enzyme was added to a cell containing buffer alone without ϵADP. The fluorescent intensity of both cells was measured and the fluorescent intensity of the second cell was subtracted from that of the first cell to eliminate contributions from protein fluorescence. Small corrections for dilution also were made. Titrations which involved high concentrations of protein, *i.e.*, the experiments to determine the stoichiometry of the ϵADP–ATPase interaction, were performed using triangular fluorescence cells to minimize the absorbance and scattering of the excitation light. In these experiments ϵADP was titrated into the protein solutions using a 10-μl Hamilton syringe with a Chaney adapter.

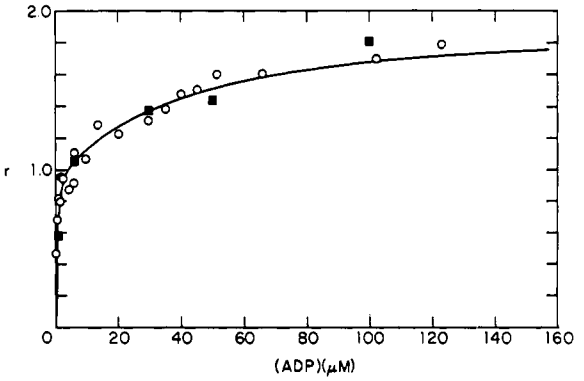

FIGURE 1: Results of the direct binding study of ADP to the solubilized ATPase in a buffer containing 0.1 M NaCl, 50 mM Tris-Cl, and 2.0 mM $MgCl_2$, pH 8.0. Data from both the gel filtration (○) and ultrafiltration (■) methods are shown. The solid line was calculated as described in the text.

Ultraviolet Difference Spectroscopy. Rectangular quartz tandem cells (Pyrocell Manufacturing Co.) having a 0.44-cm path length in each chamber were used to measure the difference spectrum resulting from the interaction of SHDP and the enzyme. The difference spectrum was recorded at room temperature on a Cary 14 recording spectrophotometer equipped with a 0–0.1 absorbance slide wire. The titration procedures have been described elsewhere (Anderson *et al.*, 1968). The concentration of SHDP was varied from 6.5×10^{-6} M to 130×10^{-6} M while the ATPase concentration was typically 6×10^{-6} M. The titration was monitored at 333 nm at a constant slit width of 0.1 mm. The pH 8.00 buffer contained 0.1 M NaCl, 50 mM Tris-Cl, and 2.0 mM $MgCl_2$.

Results and Treatment of Data

The equilibrium binding of ADP to the ATPase was studied with both the gel filtration and ultrafiltration techniques. The results in the presence of 2.0 mM Mg^{2+} are shown in Figure 1 where the number of moles of nucleotide bound per mole of enzyme, r, is plotted *vs.* the free ADP concentration. These data can be quantitatively described by assuming two independent binding sites, characterized by different binding constants, are present per enzyme molecule. In this case (Edsall and Wyman, 1958)

$$r = \frac{[\text{ADP}]}{[\text{ADP}] + K_1} + \frac{[\text{ADP}]}{[\text{ADP}] + K_2} \qquad (1)$$

The data were fit to eq 1 using a nonlinear least-squares procedure and the curve in Figure 1 has been calculated with the best fit parameters, $K_1 = 0.28 \times 10^{-6}$ M and $K_2 = 47 \times 10^{-6}$ M. The estimated experimental uncertainty for both constants is 15%.

The ultrafiltration method was selected to study the binding of ADP to the membrane-bound ATPase since small quantities of enzyme can be used, a sensitive radioactive isotope assay is possible, and a relatively short time is required for the execution of the experiment. The adenylate kinase activity of the ASU particles was found to be appreciable under the experimental conditions used so that measurements finally were carried out in the absence of a divalent metal ion and in the

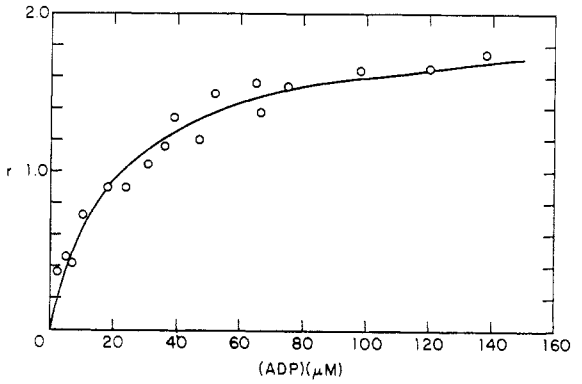

FIGURE 2: Results of the direct binding study of ADP to the solubilized ATPase in a buffer containing 0.1 M NaCl, 50 mM Tris-Cl, and 1.0 mM EDTA, pH 8.0, by the ultrafiltration method. The solid line was calculated as described in the text.

presence of EDTA, which abolished the adenylate kinase activity. The binding isotherm obtained with the solubilized enzyme in the absence of metal ion is shown in Figure 2. The data again were analyzed according to eq 1, and the curve in Figure 2 has been calculated with the best fit parameters, $K_1 = 11 \times 10^{-6}$ M and $K_2 = 43 \times 10^{-6}$ M. The estimated experimental uncertainty in these constants is 25%.

A number of ultrafiltration experiments were carried out with the membrane-bound enzyme at ADP concentrations where the solubilized ATPase bound more than 1 mol of ADP per enzyme molecule. The results obtained are summarized in Table I. In no case was a value of r greater than one found, but a quantitative binding isotherm could not be obtained. Apparently varying amounts of radioactive ADP were trapped in both the ASU particles and reconstituted particles that were layered on the filters. Since we were unable to quantitate this phenomenon, the number of binding sites present on the membrane-bound enzyme could not be calculated. However, the results obtained suggest that the very tight binding site found on the soluble enzyme either is not present or its binding affinity is considerably reduced on the membrane-bound enzyme.

In order to obtain information about the specificity of the two binding sites on the solubilized enzyme, gel filtration

TABLE I: Binding of ADP to Membrane-Bound ATPase.[a]

[ADP] (μM)	r
1.3	0.10
4.7	0.59
5.0	0.35
6.1	0.40
10	0.24
19	0.22
47	0.93
50	0.74
72	0.27
75	0.53
100	0.47

[a] Ultrafiltration experiments in pH 8.0 buffer containing 0.1 M NaCl, 50 mM Tris-Cl, and 1 mM EDTA.

TABLE II: Binding of Nucleotides to Solubilized ATPase.[a]

Nucleotide	Concn (μM)	Obsvn Wavelength (nm)	r
AMP[b]	127	259	0.2
ADP[b]	125	259	1.70
εADP[b]	83	275	1.45
IDP[b]	120	249	0.2
UDP[b]	200	262	0.2
SHDP[b]	50	313	~0.7
ATP[c]	125	259	0.2
ITP[c]	125	249	~0
UTP[c]	196	262	~0

[a] Gel filtration experiments in pH 8.0 buffer containing 0.1 M NaCl, 50 mM Tris-Cl, and either $MgCl_2$ or EDTA. [b] 2.0 mM $MgCl_2$. [c] 1.0 mM EDTA.

binding experiments were carried out with a series of nucleotides at concentrations where well over 1 mol of ADP was bound per mole of ATPase. These concentrations, in most instances, were close to the maximum permitted with the spectrophotometric monitoring used because of the high absorbancies of the solutions. When nucleotide triphosphates were studied, EDTA was substituted for Mg^{2+} in the buffer to prevent their hydrolysis (Pullman *et al.*, 1960). The results obtained are presented in Table II. Only in the case of ADP and εADP was r greater than one. The SHDP, IDP, UDP, and AMP were found to bind, but r was less than one in all instances. The result obtained with SHDP is only approximate because the absorbance peak due to the protein overlapped the absorbance trough somewhat. The ratio in Table II for SHDP was calculated by assuming that the absorbance trough extended completely through the protein peak. Although this is probably not the case, such an assumption yields a *maximum* value for r. In the absence of Mg^{2+}, r was less than one for ATP, ITP, and UTP (all substrates of the enzyme (Pullman *et al.*, 1960)). In the case of ITP and UTP overlap of the protein absorbance peak and absorbance trough was also observed although the extent of overlap was less than with SHDP. For these nucleotides the trough was not extrapolated through the protein peak so the values of r calculated are *minimum* values. For all three triphosphates the assumption was made that no hydrolysis occurred during the binding measurements (Pullman *et al.*, 1960). However, a small amount of hydrolysis might have occurred and could account for the observed binding, particularly for ATP.

The binding of ADP to the "tight" site in the presence of high concentrations of AMP, IDP, and SHDP was measured by ultrafiltration to further test the specificity of this site. The results of these experiments are shown in Table III. At an ADP concentration of 6 μM, 95% of the "tight" sites are occupied while only 10% of the "loose" sites bind ADP. High concentrations of the diphosphates, SHDP and IDP, reduce the value of r, while AMP has very little effect. If the binding of SHDP and IDP is assumed to be competitive with ADP at the "tight" site, the dissociation constants for the interaction of this site with SHDP and IDP are approximately 10 and 40 μM, respectively. These values should be considered *minimum* dissociation constants because minor impurities

TABLE III: Competition Binding Experiments.[a]

Nucleotide	Concn (mM)	r^b/r^c
AMP	1	0.94
IDP	1	0.39
SHDP	0.5	0.29

[a] Ultrafiltration experiments in pH 8.0 buffer containing 0.1 M NaCl, 50 mM Tris-Cl, 2.15 mM $MgCl_2$, 6 μM ADP, and the indicated concentration of additional nucleotide. [b] With additional nucleotide. [c] Without additional nucleotide.

(~1%) of ADP in the SHDP and IDP could cause the observed decrease in r values.

Steady-state studies of the inhibition of ATP hydrolysis by SHDP and IDP were used to investigate the interaction of these diphosphates with the catalytic site. Both SHDP and IDP are weak inhibitors of the ATPase; if competitive inhibition is assumed, inhibition constants of approximately 2 mM can be calculated.

The binding of SHDP to the "tight" site was studied further using difference spectroscopy. A typical difference spectrum obtained is shown in Figure 3. The spectrum is characterized by a peak at 333 nm, a trough at 290 nm, and an isosbestic point at 317 nm. On addition of ADP to a final concentration of 400 μM, the difference spectrum was essentially abolished as shown in Figure 3. Extreme care regarding the pH of solutions had to be used in carrying out the difference spectral measurements since the spectrum of SHDP is very sensitive to the hydrogen ion concentration at the pH where measurements were made (pH 8.0). The difference spectral titration of enzyme with SHDP is shown in Figure 4. If a single ligand binding site per enzyme molecule is assumed, then the equilibrium binding constant is

$$K = \frac{([E_0] - \Delta a/(\Delta\epsilon l))([SHDP]_0 - \Delta a/(\Delta\epsilon l))}{\Delta a/(\Delta\epsilon l)} = \frac{[E][SHDP]}{[ESHDP]} \quad (2)$$

where $[E]_0$ is the total enzyme concentration, $[SHDP]_0$ is the total SHDP concentration, Δa is the measured difference absorbance at 333 nm, $\Delta\epsilon$ is the molar difference extinction coefficient at the same wavelength, and l is the cell path length. After assuming a value of $\Delta\epsilon$, K was calculated for all SHDP concentrations using the measured values of Δa. The best value of $\Delta\epsilon$ was taken to be that which minimized the per cent standard deviation of K. The values of $\Delta\epsilon$ and K obtained are 19.1×10^3 M^{-1} cm^{-1} and 42×10^{-6} M. The minimum per cent standard deviation was 19%.

The SHTP was found to be a substrate of the ATPase, and simple Michaelis–Menten kinetics were observed over a range of SHTP concentrations from 0.16×10^{-3} M to 2.5×10^{-3} M. A Michaelis constant of 0.65×10^{-3} M was found, and the maximal velocity was about 80% of that found with ATP as the substrate. An attempt was made to utilize SHTP as an affinity label since disulfide formation with protein sulfhydryl groups can occur (Murphy and Morales, 1970). The ATPase (5 μM) and SHTP (1 mM) were incubated in a pH 8.0 buffer containing 0.025 M sucrose, 10 mM Tris-sulfate, and 1 mM EDTA. The protein and unreactive SHTP were separated by passage of the reaction mixture through a Sephadex G-25 column (1.13 $cm^2 \times 54$ cm). No increase in absorption of the protein solution was observed on addition of β-mercaptoethanol so that it was concluded affinity labeling of the enzyme did not occur.

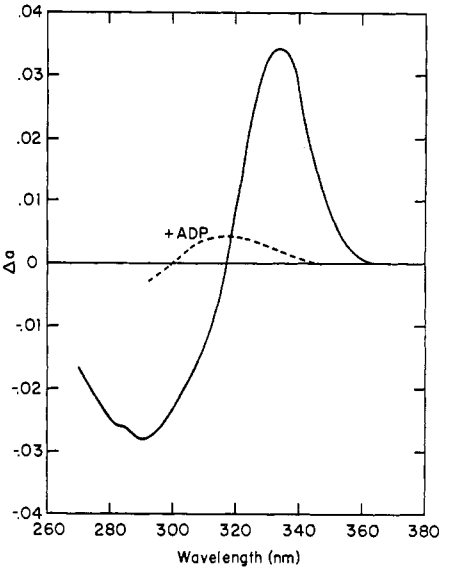

FIGURE 3: Ultraviolet difference spectrum for the interaction of SHDP with the solubilized ATPase. The buffer contained 0.1 M NaCl, 50 mM Tris-Cl, and 2.0 mM $MgCl_2$, pH 8.00: ——, 110 μM SHDP, 5.95 μM solubillzed ATPase; - - - -, 33 μM SHDP, 400 μM ADP, 5.95 μM solubilized ATPase. The cell path length was 0.44 cm.

The ϵADP is the only diphosphate studied other than ADP that binds as strongly as ADP to the "tight" site. The ϵADP–ATPase interaction was examined further by fluorescence titrations. However, the fluorescence experiments were complicated by the presence of a slow decrease in the fluorescent intensity of the ϵADP after its initial interaction with the ATPase. This slow change in fluorescence was very small and could barely be detected when small sequential additions of ϵADP or ATPase were made to each other. However, if approximately 2 μM ATPase was mixed with an equal concentration of ϵADP under very sensitive instrumental conditions,

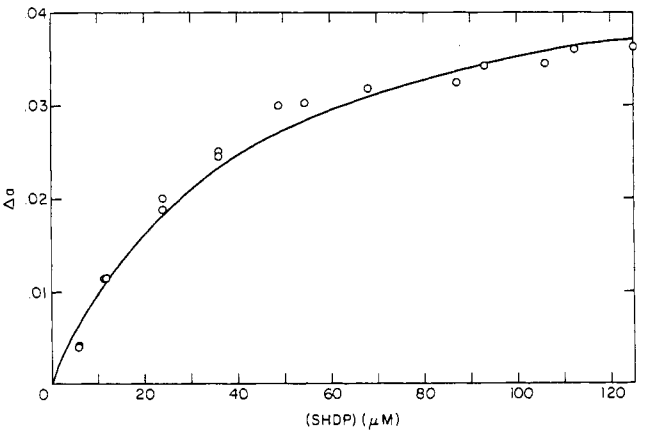

FIGURE 4: Ultraviolet difference spectrum titration of the solubilized ATPase with SHDP. The buffer contained 0.1 M NaCl, 50 mM Tris-Cl, and 2.0 mM $MgCl_2$, pH 8.00. All points have been normalized to an enzyme concentration of 5.95 μM. The solid line was calculated as described in the text. The cell path length was 0.44 cm.

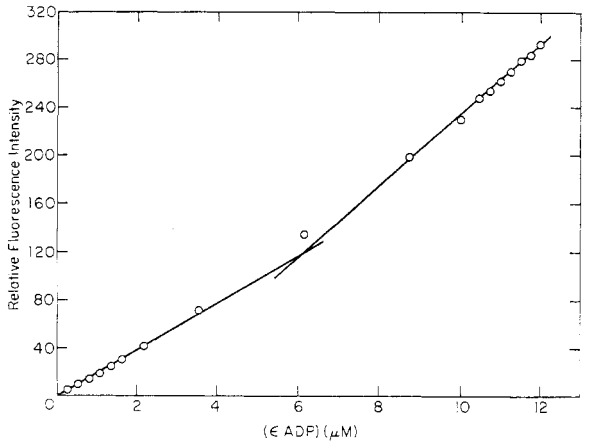

FIGURE 5: Stoichiometry of the interaction of ϵADP with solubilized ATPase as determined by fluorescence measurements. The solution contained 0.1 M NaCl, 50 mM Tris-Cl, 2.0 mM $MgCl_2$, and 6.95 μM enzyme at pH 8.0.

a slow change in fluorescence with a half-time of approximately 5 min could be readily seen. The instability of the enzyme prevented titrations from being made over a time range where the fluorescence was completely constant so that the experiments described below are only semiquantitative in nature.

The stoichiometry of binding to the "tight" site was investigated by adding ϵADP to a solution containing a concentration of enzyme much larger than the dissociation constant of the "tight" site. Under these conditions, all of the ϵADP should be complexed when its concentration is less than that of the enzyme. Thus, the observed fluorescence intensity should be proportional to the concentration of the ϵADP–ATPase complex times its quantum yield until the total ϵADP concentration exceeds the ATPase concentration. At this point the increase in the fluorescence intensity when further ϵADP is added should be proportional to the concentration of uncomplexed ϵADP times its quantum yield. The results of such a titration are shown in Figure 5. The intersection of the two straight line portions of the curve on the abscissa should be equal to the enzyme concentration if a 1:1 complex is formed. With an initial enzyme concentration of 6.95 μM (the enzyme concentration decreased about 5% during the titration due to dilution), the intersection point was 6.2 μM indicating a 1:1 complex. The change in slope of the curve indicates the fluorescence of the ϵADP is quenched when it is bound to the enzyme.

A dissociation constant was estimated for ϵADP binding to the "tight" site by titrating enzyme into a solution of ϵADP with Mg^{2+} as the divalent metal ion. Corrections were made for the fluorescence of the ATPase at each point as described in the Experimental Section. The fluorescence data were treated in a manner analogous to that for absorption difference spectra. Equation 2 was used except that instead of the term $\Delta a/(\Delta\epsilon l)$, the term $(\Delta F/\Delta F_T)[\epsilon ADP]_0$ was used, where ΔF is the observed fluorescence change and ΔF_T is the total fluorescence change when all of the ϵADP is complexed by the enzyme. A value for ΔF_T was assumed and the analysis proceeded as previously described. The value obtained for K was 0.5 μM, but this value is only approximate due to the presence of the slow fluorescence change previously described. The complexed ϵADP was found to have a fluorescence intensity which was about 30% of that of the free ϵADP.

The same fluorescence titration was carried out using Ca^{2+} as the divalent metal ion. In these experiments the total Ca^{2+} concentration was 4.5 mM which complexed about 75% of the ϵADP present. This was similar to the per cent ϵADP complexed by 2.0 mM Mg^{2+} (calculations of these percentages assumed the binding constants for Mg^{2+} and Ca^{2+} to ϵADP were the same as for binding to ADP (O'Sullivan, 1969; Walaas, 1958)). The data were treated as described above and yielded a value for the dissociation constant, K, of approximately 0.5 μM. The change in fluorescence of ϵADP on binding to the enzyme was small in the presence of Ca^{2+} (only a 5% decrease in fluorescence was observed).

An attempt also was made to detect the binding of ϵADP to the membrane-bound ATPase by fluorescence measurements. The measurements are exremely difficult because of light scattering by the membrane particles, which limited the ATPase concentration to a maximum of about 5×10^{-7} M, and because of the possible adenylate kinase activity of the membrane particles, although ϵADP has been reported not to be a substrate for rabbit muscle adenylate kinase (Secrist *et al.*, 1972). Titration experiments similar to those carried out for the soluble enzyme failed to reveal any changes in fluorescence in mixtures of ϵADP and membrane-bound ATPase. The sensitivity of detection was considerably less than in the case of the soluble enzyme because of the limitations imposed on the enzyme concentration. Therefore, the negative results obtained are only suggestive in nature and do not firmly establish that ϵADP does not interact appreciably with the membrane-bound enzyme.

Discussion

The results of the various binding studies are summarized in Table IV. These results suggest that two distinct nucleotide binding sites are present on an ATPase molecule of mol wt 285,000. One of these, the "loose" site, is clearly the catalytic site for the hydrolysis of ATP. This conclusion is supported by the fact that the dissociation constant for the interaction of ADP with the "loose" site is similar to the competitive inhibition constant (30 μM; Hammes and Hilborn, 1971) and that inhibition of the enzymatic reaction is not observed when the "tight" site is occupied by ADP, IDP, or SHDP. The existence of only one active site and two nucleotide binding sites per molecule suggests an asymmetric subunit structure for mitochondrial ATPase. The subunit structure has not yet been fully determined, but clearly is complex (Forrest and Edelstein, 1970; Senior and Brooks, 1970; Knowles and Penefsky, 1972).

The specificities of the two sites are clearly quite different. While the catalytic site binds several nucleotide mono-, di-, and triphosphates, the binding requirements for the "tight" site are very restrictive. Of the nucleotides studied, only two diphosphates, ADP and ϵADP, were capable of binding strongly to this site. The competition binding experiments suggest that SHDP and IDP, which differ only by a sulfhydryl and hydroxyl group, respectively, from ADP, were able to interact with the "tight" site, but with dissociation constants much larger than for ADP. On the other hand, ϵADP, which has a third ring system attached to the purine structure, binds quite well. This suggests that the nitrogen substituent on the 6 position of the purine ring and the diphosphate moiety of the molecule are crucial structural determinants for binding. Tight binding was not observed for ATP, ITP, and UTP in the presence of EDTA or for AMP in the presence of Mg^{2+}. The binding of diphosphates at the

TABLE IV: Equilibrium Dissociation Constants and Steady-State Kinetic Constants for Solubilized ATPase.

Nucleotide	K_1 (μM)	Exptl Technique	K_2 (μM)	Exptl Technique
ADP (with Mg^{2+})	0.28	Gel filtration[a]	47	Gel filtration[a]
			30	Steady-state kinetics[b]
ADP (with EDTA)	11	Ultrafiltration[a]	43	Ultrafiltration[a]
ϵADP (with Mg^{2+})	0.5	Fluorescent titration[a]	100–200	Estimated from gel filtration[a]
ϵADP (with Ca^{2+})	0.5	Fluorescent titration[a]		
SHDP	42	Absorbance difference spectrum titration[a]	~2000	Estimated from steady-state kinetics[b]
	>10	Competitive ultrafiltration[a]		
IDP	>40	Competitive ultrafiltration[a]	~2000	Estimated from steady-state kinetics[b]
SHTP			650	Steady-state kinetics[c]
ATP			220	Steady-state kinetics[c]

[a] pH 8.0 buffer containing 0.1 M NaCl, 50 mM Tris-Cl, and either 2 mM $MgCl_2$, 4.5 mM $CaCl_2$, or 1.0 mM EDTA. [b] pH 8.0 solution containing 0.1 M NaCl, varying amounts of nucleotide, and a free Mg^{2+} concentration maintained at 2 mM. [c] pH 8.0 solution containing 0.1 M NaCl, varying amounts of triphosphate, and a total Mg^{2+} concentration maintained at 5 mM.

catalytic site can be ordered as ADP > ϵADP > SHDP ≈ IDP ≈ UDP. The third ring system of ϵADP appears to hinder binding at the catalytic site considerably more than at the "tight" site. The substitution of a sulfhydryl group or a hydroxyl group at the 6 position of the purine ring causes even a greater perturbation in binding properties.

Another difference between the two sites is the metal ion requirement. The dissociation constants for the binding of ATP and ADP at the catalytic site do not seem to strongly depend on the presence of a divalent metal ion, whereas the dissociation constant for the binding of ADP to the "tight" site increases an order of magnitude in the absence of divalent metal. However, Ca^{2+} can replace Mg^{2+} with little alteration in the dissociation constant for the binding of ϵADP, although the quenching of fluorescence was much less in the case of Ca^{2+}.

The fact that SHTP failed to label the enzyme implies that a reactive sulfhydryl group with the correct proximity and orientation is not present at the catalytic site. The binding of various nucleotides to this site suggests that a longer "reactive arm" on the purine ring would provide a better affinity label. The great difference in the specificity of the two sites should make it possible to selectively label them.

Unfortunately detailed binding studies could not be carried out with the membrane-bound enzyme. The evidence obtained suggests the "tight" site may not be present or may have a reduced binding affinity when the enzyme is bound to the membrane. Significant differences between the properties of the solubilized and membrane-bound enzyme are known to exist: for example, the kinetic parameters and ADP inhibition (Hammes and Hilborn, 1971), oligomycin inhibition (Racker, 1962), and cold lability (Pullman *et al.*, 1960).

The function of the "tight" site on the solubilized enzyme is unknown, although its great specificity suggests one must exist. Binding of ADP to the tight site may be related to the ADP–ATPase complex observed by Zalkin *et al.* (1965). Perhaps, as suggested by the failure to observe it on the membrane-bound enzyme, it is involved in the membrane–enzyme interaction. Alternatively, it may be an allosteric control site: although binding at the "tight" site has not been found to be inhibitory, experiments have not been done at ATP concentrations much smaller than the ATP Michaelis constant. For the chloroplast ATPase, ADP has been found to be an allosteric effector (Nelson *et al.*, 1972). The "tight" site also might be part of the dinitrophenol binding site or of the oligomycin binding site on the membrane-bound enzyme. These possibilities are currently under further investigation. In any event, a more complete characterization of mitochondrial ATPase on and off the membrane should provide further information about the influence exerted by a natural membrane on the properties of enzymes bound to it.

References

Anderson, D. G., Hammes, G. G., and Walz, F. G., Jr. (1968), *Biochemistry 7*, 1637.

Barrio, J. R., Secrist, J. A., III, and Leonard, N. J. (1972), *Biochem. Biophys. Res. Commun. 46*, 597.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Datta, A., and Penefsky, H. S. (1970), *J. Biol. Chem. 245*, 1537.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, Vol. 1, New York, N. Y., Academic Press, p 613.

Forrest, G., and Edelstein, S. J. (1970), *J. Biol. Chem. 245*, 6468.

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

Hampton, A., and Maguire, M. H. (1961), *J. Amer. Chem. Soc. 83*, 150.

Horstman, L. L., and Racker, E. (1970), *J. Biol. Chem. 245*, 1336.

Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63*, 530.

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

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

Knowles, A. F., and Penefsky, H. S. (1972), *J. Biol. Chem. 247*, 6617, 6624.

MacLennan, D. H., and Tzagoloff, A. (1968), *Biochemistry 7*, 1603.

Murphy, A. J., Duke, J. A., and Stowring, L. (1970), *Arch. Biochem. Biophys. 137*, 297.

Murphy, A. J., and Morales, M. F. (1970), *Biochemistry 9*, 1528.

Nelson, N., Nelson, H., and Racker, E. (1972), *J. Biol. Chem. 247*, 6506.

O'Sullivan, W. J. (1969), *in* Data for Biochemical Research,

Dawson, R. M. C., Elliot, D. C., Elliot, W. H., and Jones, K. M., Ed., New York, N. Y., Oxford University Press, p 433.
Paulus, H. (1969), *Anal. Biochem. 32*, 91.
Penefsky, H. S., Pullman, M. E., Datta, A., and Racker, E. (1960), *J. Biol. Chem. 235*, 3330.
Penefsky, H. S., and Warner, R. C. (1965), *J. Biol. Chem. 240*, 4694.
Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960), *J. Biol. Chem. 235*, 3322.
Racker, E. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1659.
Racker, E., and Horstman, L. L. (1967), *J. Biol. Chem. 242*, 2547.
Secrist, J. A., III, Barrio, J. R., Leonard, N. J., and Weber, G. (1972), *Biochemistry 11*, 3499.
Senior, A. E., and Brooks, J. C. (1970), *Arch. Biochem. Biophys. 140*, 257.
Walaas, E. (1958), *Acta Chem. Scand. 12*, 528.
Zalkin, H., Pullman, M. E., and Racker, E. (1965), *J. Biol. Chem. 240*, 4011.

Glycosyltransferase and Sialic Acid Levels of Normal and Transformed Cells†

William J. Grimes‡

ABSTRACT: Ability to catalyze transfer of sialic acid, fucose, and galactose to exogeneous acceptors was measured in extracts from a wide variety of normal and transformed cells. Sialic acid transferring ability was reduced when normal mouse cells are transformed by Simian virus 40 (SV40), and hamster cell lines are transformed by polyoma virus (Py) or hamster sarcoma virus. Flat revertants of SV40-transformed cells have increased while spontaneous transformed cells have decreased sialic acid transferring ability. Py virus transformed mouse cells were the only transformed cell line which did not have decreased sialic acid transferring ability. Galactose and fucose transferring ability was reduced when some cell lines were transformed, but was unaltered in several others. Sialic acid levels of all cell lines were found to be directly related to sialic acid transferring ability measured in cell extracts.

The cell surface has long been postulated to function in the regulation of cell growth. A correlate of this postulate is that cell surface alterations will lead to loss of the regulatory control of cell division. Biochemical studies of cell surface glycoproteins and glycolipids have revealed alterations associated with transformation.

Experiments measuring relative compositions of glycoproteins from 3T3 and SV40-transformed 3T3 cells indicate reduced carbohydrate levels in the transformed cells (Wu *et al.*, 1969). Chemical measurements show that cells transformed by RNA and DNA viruses have reduced levels of sialic acid (Ohta *et al.*, 1968; Grimes, 1970; Perdue *et al.*, 1972). Evidence has been published that glycoprotein structures are altered by transformation (Meezan *et al.*, 1969; Buck *et al.*, 1971; Onodera and Sheinin, 1970). Biochemical studies on glycolipids have shown that in hamster cells, hematoside, the Forssman antigen, and ceramide trihexoside are reduced in transformed cells as compared with normal cells (Hakamori and Murakami, 1968; Hakomori, 1970; Robbins and Macpherson, 1971). In cultured cells derived from mouse embryos, the complex gangliosides of normal cells are lacking in transformed cells (Mora *et al.*, 1969).

Alterations in complex carbohydrate compounds are accompanied by changes in the glycosyltransferases. Transformed hamster cells have reduced levels of the α-galactosyltransferase which catalyzes synthesis of ceramide trihexoside (Kijunoto and Hakomori, 1971). Transformed mouse cells have reduced levels of a *N*-acetyl-D-galactosaminyltransferase which catalyzes synthesis of Tay Sachs ganglioside (Cumar *et al.*, 1970). Altered transferase levels accompany cell density dependent changes in glycolipid patterns (Hakomori, 1970; Robbins and Macpherson, 1971; Kijunoto and Hakomori, 1971). Bosmann *et al.* (1968a,b) reported increased glycoprotein glycosyltransferase activities in transformed cells. The ability to catalyze sialic acid transfer to hematoside, monosialyl ganglioside, and desialized glycoprotein acceptors has been reported to be decreased in transformed cells (Grimes, 1970; Den *et al.*, 1971; Grimes and Robbins, 1972). Warren *et al.* (1972) have observed that ability to catalyze sialic acid transfer to fucose containing glycopeptides is elevated in transformed cells. To determine the generality of some of these observations we have studied sialic acid, galactose, and fucose transferases and sialic acid levels in a large variety of normal and transformed cells.

Materials and Methods

Cell Lines. Cell lines used in experiments described have the following origin: 3T3B, SV3T3B, were a gift of Dr. Paul Black, Massachusetts General Hospital. 3T3B is a cloned line of the original Swiss 3T3 cells (3T3G) from Howard Green (see below). SV3T3B is a cell line derived from the cloned line 3T3B after transformation by SV40. ST3T31B is a spontaneous transformant of 3T3B which is less sensitive to contact inhibition. ST3T32B is a second spontaneous transformant cell line and was a gift of Dr. Loyd Culp at Massachusetts General

† From the Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85721. *Received June 29, 1972.* Supported by Grant CA 12753 from the National Cancer Institute.