Activation of Beef Heart Mitochondrial Adenosine Triphosphatase by 2,4-Dinitrophenol†

Lewis C. Cantley, Jr.,‡ and Gordon G. Hammes*

ABSTRACT: Initial velocity measurements of the hydrolysis of ATP catalyzed by both solubilized and reconstituted beef heart mitochondrial ATPase have been carried out in the presence of 2,4-dinitrophenol with the pH stat technique at pH 8.0, 0.1 M NaCl, and 5.0 mM Mg^{2+}. For both the solubilized and reconstituted enzymes, dinitrophenol activates the enzyme by lowering the apparent ATP Michaelis constant. At dinitrophenol concentrations up to 0.30 mM, the maximum velocity of the soluble enzyme slightly increases, while that of the membrane-bound enzyme shows little change. For higher dinitrophenol concentrations, the maximum velocity decreases for both forms of the enzyme. The equilibrium binding of dinitrophenol to the solubilized ATPase also was studied. A new "forced" dialysis technique was utilized which is more rapid and convenient than equilibrium dialysis, gel filtration, or ultrafiltration. Moreover, the precision obtained is as good as with any of the conventional methods. The results obtained indicate that at high dinitrophenol concentrations (>0.3 mM), a large amount of nonspecific binding occurs, causing enzyme denaturation. The dinitrophenol binding is not competitive with the binding of ADP, indicating dinitrophenol is not directly interacting with the active site. Either one or two tight dinitrophenol binding sites are responsible for activation of the enzyme. A mechanism is proposed, involving the binding of dinitrophenol to a single activating site, which quantitatively accounts for the activation of the enzyme. The primary difference between the solubilized and reconstituted enzymes is that dinitrophenol binds more strongly to the solubilized enzyme.

The Mg^{2+} dependent cold-labile ATPase from beef heart mitochondria is postulated to play an important role in oxidative phosphorylation. Extensive chemical and physical studies of this enzyme have been carried out (*cf.* Horstman and Racker, 1970; MacLennan and Tzagoloff, 1968). It has a complex subunit structure and contains five different types of subunits (Knowles and Penefsky, 1972). This ATPase also represents an important model for the study of enzyme–membrane interactions. The soluble enzyme can be spontaneously recombined with native membrane fragments (ASU particles) devoid of ATPase activity (Penefsky *et al.*, 1960). The kinetic properties of the soluble and reconstituted membrane-bound enzyme differ (Hammes and Hilborn, 1971), and the reconstituted enzyme behaves similarly to the native mitochondrial-bound enzyme (Penefsky *et al.*, 1960). The enzyme is activated by 2,4-dinitrophenol, which is also an uncoupler for oxidative phosphorylation (Pullman *et al.*, 1960).

In this work, equilibrium binding and steady-state kinetics experiments have been used to investigate the activation of solubilized and reconstituted mitochondrial ATPase by 2,4-dinitrophenol. In both cases, the activation involves a lowering of the Michaelis constant; the maximum velocity also is increased in the case of the solubilized enzyme. A simple activation mechanism adequately accounts for all of the data. In addition, a "forced dialysis" technique is described which permits precise binding studies to be made very rapidly.

Experimental Section

Materials. The 2,4-dinitrophenol was purchased from Eastman Kodak Co. and was recrystallized from methanol–water. The final product had a melting point of 112–113°. The concentration of the stock dinitrophenol solution was determined by measurement of the absorbance at 360 nm at pH 11, using an extinction coefficient of $1.48 \times 10^4\ M^{-1}\ cm^{-1}$ (Phillips *et al.*, 1962). The ATP and ADP were obtained from Sigma Chemical Co. Sephadex was purchased from Pharmacia Fine Chemical Co. The [^{3}H]ADP (5–15 Ci/mmol) and [^{14}C]-2,4-dinitrophenol (6.3 Ci/mol) were obtained from New England Nuclear. The [^{3}H]ADP was purified by paper chromatography using the solvent system isobutyric acid–1 N ammonia (100:60 v/v). All other chemicals were the best available commercial grades, and deionized distilled water was used to prepare all solutions.

ATPase. The preparation of ATPase and ASU membrane particles from beef heart mitochondria followed known procedures (Horstman and Racker, 1970; Racker and Horstman, 1967). The beef heart mitochondria were generously supplied by Dr. E. Racker. A final step, previously described, was used to eliminate turbidity in the enzyme solution (Hilborn and Hammes, 1973). The ATPase was stored as an ammonium sulfate precipitate at 4°. The specific activity of the enzyme was 90–100 μmol/(mg min). Activity assays and steady-state velocities were determined using the pH stat technique (Hammes and Hilborn, 1971). The assay conditions were as follows: 5 mM $MgCl_2$, 0.1 M NaCl, *ca.* 0.3 μg/ml of ATPase, 2.60 mM ATP, pH 8.0, 25°. The titration of protons formed by the reaction was done with 1–2.5 mM NaOH.

Protein concentrations were determined by the method of Lowry *et al.* (1951) using four times crystallized human serum albumin (Nutritional Biochemicals Corp.) as the standard. The resultant concentration was divided by 1.18 to obtain the dry weight of the enzyme (Kagawa and Racker, 1966). A molecular weight of 285,000 (Forrest and Edelstein, 1970; Penefsky and Warner, 1965) was used to determine molar concentrations of enzyme. This molecular weight is characteristic of the enzyme prepared by the methods described

† From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received July 10, 1973.* This work was supported by a grant from the National Institutes of Health (GM 13292).

‡ National Science Foundation Predoctoral Fellow.

here, although higher molecular weights have been reported for other preparations (Knowles and Penefsky, 1972).

Membrane-bound ATPase was reconstituted as described by Hammes and Hilborn (1971). The specific activity of the reconstituted enzyme was based on the amount of soluble ATPase bound rather than on the total protein concentration. The specific activity was determined by measuring the activity of the reconstituted enzyme and comparing it, under identical conditions, with the specific activity of a soluble ATPase solution of equal enzyme concentration. The specific activity of membrane-bound ATPase was approximately 60 μmol/(mg min).

Kinetic Measurements. Initial velocities were measured with the pH stat technique at pH 8.0 in 0.1 N NaCl, 5 mM $MgCl_2$, and varying amounts of ATP and dinitrophenol. In initial experiments the concentration of $MgCl_2$ was varied to keep the concentration of free Mg^{2+} constant, but it was discovered that the initial velocity was not sensitive to free Mg^{2+} concentrations of less than 5 mM as long as the reaction was started by adding the enzyme to the equilibrated MgATP solution. The ATPase–ammonium sulfate suspension was dissolved in buffer (0.025 M sucrose–1 mM EDTA–10 mM Tris chloride (pH 8.4)) at room temperature and ATP (1 mM) was added to stabilize the enzyme. A 15- or 20-μl aliquot of this solution was then added to the reaction mixture (final volume 4 ml) to initiate the reaction. Both the soluble ATPase and reconstituted ATPase–buffer solutions were used within 8 hr after preparation and were constant in activity during this time. The rate of change of pH due to CO_2 absorption was less than 2% of the measured ATPase rates. The standard deviation in velocities varied from about $\pm 3\%$ at high ATP concentrations ($\sim$2.5 mM) to about $\pm 15\%$ at low ATP concentrations ($\sim$0.10 mM). All measurements were made at least in duplicate.

Binding Measurements. The binding of dinitrophenol to soluble ATPase was initially studied using the ultrafiltration method (Paulus, 1969). The ultrafiltration cell was obtained from Metalloglass, Inc., Boston, Mass. Diaflo XM-50 membranes were found to be suitable since all the ATPase was retained and only 1–2 μl of the total volume of [^{14}C]dinitrophenol was left on the membrane in the absence of protein. The dinitrophenol binds tightly to the ionic UM-10 membranes rendering them unsuitable. Quadruplicate determinations were done for all dinitrophenol concentrations both with and without protein. The [^{14}C]dinitrophenol–ATPase–buffer solution was mixed in 1–2-ml portions and 200-μl aliquots were added to each channel of the ultrafiltration cell with a resulting total quantity of 50–100 μg of ATPase in each channel. The buffer used in all binding experiments contained 50 mM Tris chloride–0.1 M NaCl–2 mM $MgCl_2$ at pH 8.0 and room temperature. In order to remove ATP and ADP from the ATPase–ammonium sulfate precipitate, the enzyme was passed through a Sephadex G-25 (medium) column (Hilborn and Hammes, 1973) before being dissolved in the buffer. Although the ammonium sulfate precipitated enzyme is stable at 4° in the absence of ATP (Hilborn and Hammes, 1973), an exponential decay in activity of the enzyme occurs in Mg^{2+}–Tris chloride buffer in the absence of ATP with a reaction half-time of about 40 min. Similar losses of activity were previously observed with both beef heart and rat liver mitochondrial ATPase (Chang and Penefsky, 1973; Catterall and Pedersen, 1972). Since the filtration time was usually 1–2 hr, the enzyme retained only 25–30% of its full activity at the end of the experiment. When Diaflo XM-300 membranes (which allow a small quantity of enzyme to pass through) were used with filtering times of 5–10 min, nearly identical results were obtained indicating a change in binding properties of the enzyme did not accompany the activity loss. A pressure of 40 psi of nitrogen was applied until the filtration was complete, and the undersides of the membranes were then washed with 5 ml of ethylene glycol. The membranes were placed in scintillation vials containing 0.25 ml of 2 M NaOH and allowed to stand overnight. The NaOH was titrated to a clear end point with BBS-2 Beckman Solubilizer and 10 ml of Beckman-Toluene Fluor scintillation fluid was added. The radioactivity was assayed in a Beckman LS-255 liquid scintillation counter.

Because of the instability of the enzyme and the irreproducibility of measurements at high enzyme and dinitrophenol concentrations, a new method was devised to carry out equilibrium binding measurements. Equilibrium dialysis requires many hours for the membrane equilibrium to be established; however, chemical equilibration at concentrations near the dissociation constant is usually quite fast (less than 30 min). If a very small portion of this equilibrated solution is forced through a membrane which retains the protein but allows free passage of the substrate, it is possible to measure the free ligand concentration; the total ligand concentration can be readily measured in the ligand–protein solution. The Metalloglass ultrafiltration cell proved to be adequate for this "forced dialysis" technique. The ligand–enzyme–buffer solution was mixed in 0.5-ml portions and 0.2-ml aliquots were added to two channels in the ultrafiltration cell. The total ligand concentration was determined by measuring the radioactivity of 10-μl aliquots in 0.2 ml of water and 10 ml of scintillation fluid as previously described. After a short chemical equilibration time (*ca.* 20 min), 25 psi of nitrogen was applied for about 5 min until 10 μl of solution was forced through the membrane. The 10-μl aliquots were collected by inserting Microcap pipets through the capillary holes in the cell used for washing the undersides of membranes (20 μl or larger samples may be collected from the bottom of the cell). These 10-μl aliquots were added to scintillation vials containing 0.2 ml of water, a solubilizer, and scintillation fluid. The Diaflo XM-50 membranes were used; much less porous membranes may be used for this technique since filtering time is not important. As a control, several measurements were made in the absence of enzyme to ensure that the membrane did not retain or retard passage of the ligand. As in dialysis measurements, it is necessary to adjust the enzyme and ligand concentrations to levels comparable to the dissociation constant if good precision is to be obtained. The experimental error in determining the free and total ligand concentration is approximately $\pm 3\%$, and the protein is only concentrated about 5% during the course of the experiment.

Results and Treatment of Data

In order to determine the concentrations of dinitrophenol causing activation of the ATPase, a series of initial velocities was measured using the pH stat technique. The reactions were carried out under the assay conditions described previously, and the ATP concentration was held constant while the dinitrophenol concentration was varied. The results are shown in Figure 1 for two concentrations of ATP. In this figure the per cent activation (0% represents no activation) is plotted against the logarithm of the dinitrophenol concentration. The data indicate that activation occurs at low dinitrophenol concentrations, while inhibition occurs at high concentrations, with a maximum activation occuring at about 0.3 mM dinitrophenol.

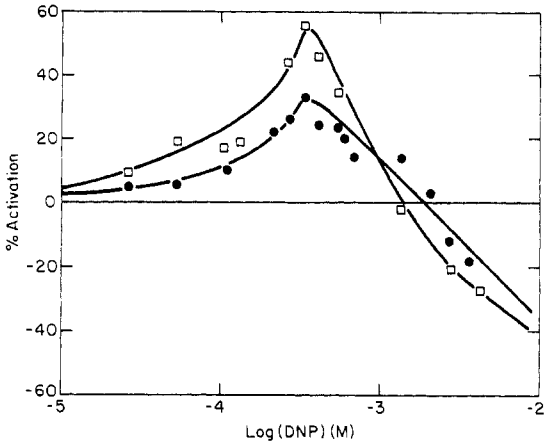

FIGURE 1: A plot of the percentage change in the initial velocity *vs.* the concentration of dinitrophenol at pH 8.0, 0.1 M NaCl, 5.0 mM Mg^{2+}, and 25°, with the initial concentrations of ATP fixed at 0.5 mM (□) and 2.5 mM (●).

The equilibrium binding of dinitrophenol to ATPase in the presence of 2.0 mM Mg^{2+} was studied using both the ultrafiltration and forced dialysis techniques. Identical results were obtained before and after passage of the enzyme through a Sephadex column; the column eliminates residual amounts of nucleotide. The results reported here were obtained without Sephadex treatment of the enzyme. The ultrafiltration method was found to give good results at low protein and dinitrophenol concentrations, but at high concentrations irreproducible results and unreasonably large amounts of ligand bound were found. This is due to the fact that the amount of protein precipitating out of solution is sufficiently great to trap dinitrophenol, suggesting erroneously large amounts of ligand are bound. We have observed this effect in other systems also. The forced dialysis technique, on the other hand, gave very reproducible results over a wide range of ligand and protein concentrations. The results of the forced dialysis experiments are summarized in the Scatchard plot in Figure 2 where *r*/[dinitrophenol] is plotted *vs.* the free dinitrophenol concentration; *r* is the number of moles of ligand bound per mole of protein.

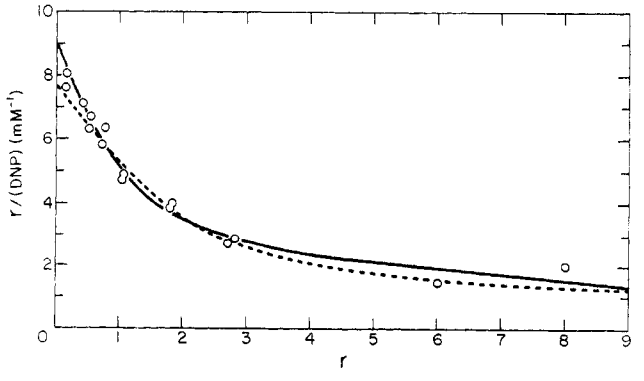

FIGURE 2: A plot of *r*/(Dnp) *vs.* *r* for the solubilized ATPase in 0.1 M NaCl–50 mM Tris chloride–2 mM Mg^{2+} (pH 8.0) at 25°. Here *r* is the number of moles of dinitrophenol bound per mole of enzyme and (Dnp) is the dinitrophenol concentration. Measurements were made by the forced dialysis technique. The solid curve was calculated with eq 1 using the best fit parameters $n_1 = 1$, $n_2 = 18$, $K_1 = 6.91$ mM^{-1}, and $K_2 = 0.124$ mM^{-1}; the dashed curve was calculated with eq 1 using the best fit parameters $n_1 = 2$, $n_2 = 30$, $K_1 = 3.20$ mM^{-1}, and $K_2 = 0.04$ mM^{-1}.

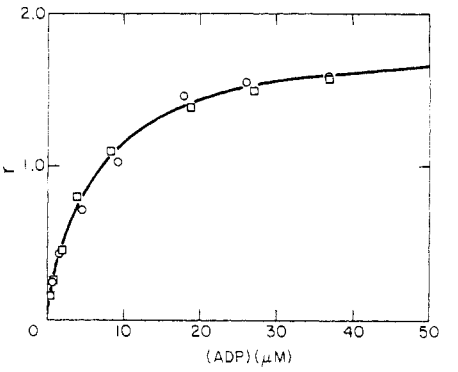

FIGURE 3: A plot of *r vs.* (ADP) where *r* is the number of moles of ADP bound per mole of enzyme. The buffer contained 0.1 M NaCl–50 mM Tris–2 mM Mg^{2+} (pH 8.0) at 25°; (□) 0.302 mM dinitrophenol; (○) no dinitrophenol. The forced dialysis method was used.

The data were fit to eq 1 in which two types of binding sites are assumed, namely activator and inhibitor sites as suggested by the steady-state kinetic data. Here n_1 and n_2

$$r = \frac{n_1K_1(\text{Dnp})}{1 + K_1(\text{Dnp})} + \frac{n_2K_2(\text{Dnp})}{1 + K_2(\text{Dnp})} \quad (1)$$

are the number of binding sites per enzyme molecule of each class, K_1 and K_2 are the corresponding binding constants, and (Dnp) is the dinitrophenol concentration. A nonlinear least-squares program was used where n_2, K_1, and K_2 were taken as unknowns and n_1 was assumed to be 1 or 2. (The assumption that $n_1 = 3$ gave a very poor fit to the data.) The best fit sets of parameters obtained, requiring n_2 to be an integer, are $n_1 = 1$, $n_2 = 18$, $K_1 = 6.91$ mM^{-1}, $K_2 = 0.124$ mM^{-1}, and $n_1 = 2$, $n_2 = 30$, $K_1 = 3.20$ mM^{-1}, and $K_2 = 0.04$ mM^{-1}. The solid curve in Figure 2 was calculated with eq 1 and the first set of parameters, while the dashed curve was calculated with eq 1 and the second set. For both sets of parameters, the uncertainty in K_1 is about ±10% and the uncertainty in K_2 is about ±70%. In either case, n_1K_1 is about 6.6 mM^{-1}.

To further characterize the dinitrophenol binding sites, the binding of ADP to solubilized ATPase was studied in the presence of 0.302 mM dinitrophenol. This study was prompted by evidence that ADP binds very tightly to a site whose function has not yet been established (Hilborn and Hammes, 1973). The "forced dialysis" technique was used and nucleotides were removed from the ATPase by Sephadex chromatography as described in the Experimental Section. Diaflo XM-50 membranes and [^{3}H]ADP were used. The results are shown in Figure 3, where the number of moles of bound ADP per mole of ATPase, *r*, in the absence and presence of 0.302 mM dinitrophenol, is plotted *vs.* the concentration of free ADP. The presence of 0.302 mM dinitrophenol has no discernible effect on the binding of ADP, indicating that the ligands are not competitive for the same binding sites. The binding isotherm for ADP is identical with that obtained with other methods (Hilborn and Hammes, 1973), thus providing a good check of the forced dialysis technique.

A series of initial velocities was measured using the soluble enzyme by the pH stat technique where the dinitrophenol concentration was held constant and the ATP concentration varied from 0.10 to 5.2 mM. Plots of $1/v$ *vs.* 1/[ATP], where v is the initial velocity, yielded straight lines for all dinitrophenol concentrations. The apparent maximum velocities,

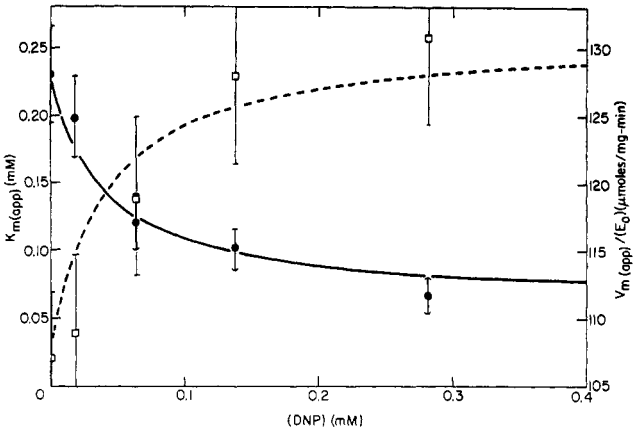

FIGURE 4: A plot of the apparent Michaelis constant $K_{m(app)}$ (●) and turnover number $V_{m(app)}/(E_0)$ (□) *vs.* the dinitrophenol concentration for the solubilized ATPase. The solid and dashed lines were calculated using eq 3 and the parameters K_m = 0.23 mM, K_m' = 0.062 mM, K_1 = 6.91 mM^{-1}, k_3 = 107 μmol/(mg min) and k_6/k_3 = 1.22. The parameters were obtained as described in the text. A weighted least-squares fit of $1/v$ *vs.* 1/(ATP) at constant dinitrophenol concentrations, pH 8.0, 5 mM $MgCl_2$–0.1 M NaCl, 25°, was used to obtain the apparent steady-state constants.

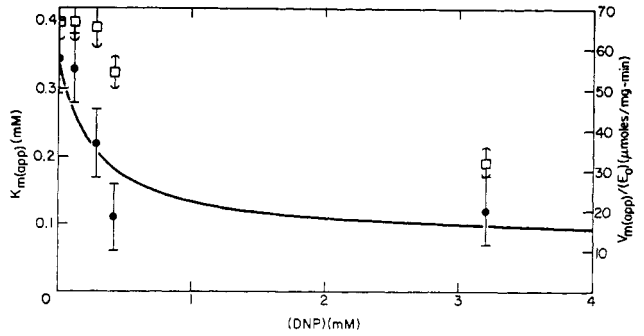

FIGURE 5: A plot of the apparent Michaelis constant $K_{m(app)}$ (●) and turnover number $V_{m(app)}/(E_0)$ (□) *vs.* dinitrophenol concentration for the membrane-bound enzyme. The solid line was calculated using eq 3 and the parameters K_m = 0.345 mM, K_m' = 0.076 mM, and K_1 = 0.77 mM^{-1}. The parameters were obtained as described in the text. A weighted least-squares fit of $1/v$ *vs.* 1/(ATP) at constant dinitrophenol concentrations, pH 8.0, 5 mM $MgCl_2$–0.1 M NaCl, 25°, was used to obtain the apparent steady-state constants.

$V_{m(app)}$, and Michaelis constants, $K_{m(app)}$, which are presented in Figure 4, were determined by a weighted least-squares analysis of the double reciprocal plots. The uncertainty is ±15% in $K_{m(app)}$ and ±5% in $V_{m(app)}$. For dinitrophenol concentrations up to about 0.3 mM, the apparent Michaelis constant decreases and the apparent maximum velocity increases. For higher dinitrophenol concentrations, the apparent maximum velocity begins to decrease and the apparent Michaelis constant remains at its minimum value. Because of the scattered low velocities and the indication of numerous weak binding sites for dinitrophenol concentrations above 0.3 mM, the decrease in the maximum velocity at high dinitrophenol concentrations is attributed to nonspecific binding causing denaturation. In proposing a mechanism for activation, the inhibition was ignored and only data for dinitrophenol concentrations below 0.3 mM were used.

A simple mechanism accounting for the observed activation is given in eq 2. Here E is the enzyme, P_i is inorganic

$$\begin{gathered} E + ATP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}ATP \xrightarrow{k_3} E + ADP + P_i \\ E + Dnp \overset{K_1}{\rightleftharpoons} Dnp\text{-}E \qquad (2) \\ Dnp\text{-}E + ATP \underset{k_6}{\overset{k_4}{\rightleftharpoons}} Dnp\text{-}E\text{-}ATP \xrightarrow{k_6} Dnp\text{-}E + ADP + P_i \end{gathered}$$

phosphate, the lower case k's are rate constants, the upper case K is an equilibrium constant, and a single dinitrophenol site has been assumed. The initial velocity, v, for this mechanism, using the usual steady-state assumption, is given by eq 3. Here (E_0) is the total enzyme concentration, $K_m = (k_2 + k_3)/k_1$, $K_m' = (k_6 + k_5)/k_4$, and $V_{m(app)}$ and $K_{m(app)}$ are defined by eq 3. Equation 3 predicts the usual linear double reciprocal plot of $1/v$ *vs.* 1/(ATP) at constant dinitrophenol concentrations, with the apparent maximum velocity and the apparent Michaelis constant being functions of the dinitrophenol concentration. In order to reduce the number of unknown parameters, K_m and k_3 were determined in the absence of dinitrophenol and the value of K_1 was assumed to be 6.91 mM^{-1}, as determined from the equilibrium binding measurements. Since the most pronounced effect of dinitrophenol on the soluble enzyme is a lowering of the apparent K_m, the parameter K_m' was determined from a least-squares fit of the dinitrophenol dependence of the apparent Michaelis constants in Figure 4. The best fit was obtained with K_m' = 0.062 mM, with an uncertainty of ±12%. A value of k_6/k_3 = 1.22 (±3%) was determined by fitting the variation of the apparent maximum velocity with dinitrophenol concentration and assuming K_m' = 0.062 mM. Figure 4 shows a plot of $K_{m(app)}$ and $V_{m(app)}/(E_0)$ *vs.* the dinitrophenol concentration. The lines represent the theoretical curves obtained using eq 3 and the parameters given above.

$$v = \frac{k_3(E_0)\left[\dfrac{1 + K_1(k_6/k_3)(K_m/K_m')(Dnp)}{1 + K_1(K_m/K_m')(Dnp)}\right]}{1 + \dfrac{K_m}{(ATP)}\left[\dfrac{1 + K_1(Dnp)}{1 + K_1(K_m/K_m')(Dnp)}\right]} = \frac{V_{m(app)}}{1 + K_{m(app)}/(ATP)} \qquad (3)$$

A similar set of initial velocity measurements was conducted using the reconstituted ATPase. Again, double reciprocal plots yielded straight lines, and a lowering of the apparent Michaelis constant with increasing dinitrophenol concentration occurred. However, the apparent maximum velocity remained constant for dinitrophenol concentrations up to 0.3 mM, and decreased at higher dinitrophenol concentrations. The apparent Michaelis constants and maximum velocities are presented in Figure 5. The dinitrophenol dependence of the values of $K_{m(app)}$ in Figure 5 was fit to the mechanism of eq 2 as before except that both K_1 and K_m' were allowed to vary and K_m was found to be 0.34 mM in the absence of dinitrophenol. The best fit yielded the values K_1 = 0.77 mM^{-1} and K_m' = 0.076 mM. The uncertainty in each parameter holding the other constant is 10–20%, but with both parameters varying, the uncertainties are somewhat larger. The solid line in Figure 5 is a theoretical curve of $K_{m(app)}$ obtained using the above parameters. Since there was no change in $V_{m(app)}$ at dinitrophenol concentrations below 0.3 mM, k_6 and k_3 are assumed to have approximately the same values (67 μmol/(mg min)), and the lowering of $V_{m(app)}$ at high dinitrophenol concentrations is again attributed to nonspecific binding.

Discussion

The results obtained establish that dinitrophenol is both an activator and inhibitor of the solubilized and reconstituted ATPase. Both the steady-state kinetics and binding experiments indicate that the inhibition is noncompetitive in nature. Dinitrophenol, at a concentration of 0.3 mM, has no effect on the binding of ADP to either the active site or the "tight" site, whose function has not yet been established (Hilborn and Hammes, 1973). At the dinitrophenol concentrations causing inhibition, many dinitrophenol molecules are bound per enzyme molecule suggesting nonspecific binding occurs which causes denaturation of the enzyme. At the dinitrophenol concentrations causing maximal activation of the soluble enzyme, however, only a few dinitrophenol molecules are bound per enzyme molecule, and the detailed analysis of the binding experiments suggests that the binding of only 1 or 2 molecules of dinitrophenol causes activation.

Phenomenologically the activation of the enzyme by dinitrophenol results in a marked decrease in the apparent Michaelis constant for ATP. The apparent maximal velocity is increased slightly for the solubilized enzyme and remains about constant for the reconstituted enzyme. Since the apparent Michaelis constant for ATP decreases, the apparent maximum velocity for ATP hydrolysis is about constant, and the binding constant for ADP is unchanged by dinitrophenol, the relationship between the equilibrium constant and the steady-state kinetic parameters requires that the maximum velocity for the synthesis of ATP from ADP and P_i increases and/or the Michaelis constant for phosphate decreases. The simple mechanism proposed for the activation by dinitrophenol in eq 2 is, of course, not unique, but it quantitatively accounts for changes in both the maximum velocity and the Michaelis constant. More complex mechanisms are also possible. For example, if dinitrophenol also binds to the enzyme–ATP complex, the form of the rate equation is identical with eq 3, assuming the dinitrophenol binding reactions are at equilibrium during the course of the reaction. If two molecules of dinitrophenol are assumed to be involved in the activation process, an additional adjustable parameter appears in the expression for the initial velocity, which permits a slightly better fit of the data to be made. At the present time it is not possible to determine whether one or two specific activation binding sites exist for dinitrophenol. The complexity of the subunit structure of the enzyme precludes any reasonable estimate on a structural basis (Knowles and Penefsky, 1972).

Although both the solubilized and reconstituted enzymes are activated by dinitrophenol, dinitrophenol binds considerably tighter to the former than to the latter (the binding constants are 6.9 and 0.77 mM^{-1}, respectively). The weak binding of dinitrophenol to the reconstituted enzyme made it particularly difficult to investigate the activation mechanism for this form of the enzyme. The biological significance of the dinitrophenol control site is unknown, but it is not unreasonable to assume a dinitrophenol-like activator exists in the mitochondria. Whether the enzyme activation is related to the uncoupling of oxidative phosphorylation also remains to be determined, although it would seem quite coincidental for dinitrophenol to possess two such specific, but unrelated, functions.

Finally, the "forced" dialysis technique used for binding measurements should be noted. This technique is much more convenient and rapid than conventional equilibrium dialysis, gel filtration, and ultrafiltration techniques. Small volumes are used and it is only necessary to wait long enough to establish chemical equilibrium, thus eliminating the long time required to establish membrane equilibrium or to elute a column. The original protein solution is concentrated a very small amount, about 5%, so that the binding of ligands to aggregating protein systems also can be readily studied with this method. Very precise results can be obtained: the precision is comparable to that obtained with equilibrium dialysis and gel filtration and is better than that obtained with ultrafiltration.

References

Catterall, W. A., and Pedersen, P. L. (1972), *J. Biol. Chem. 247*, 7969.

Chang, T., and Penefsky, H. S. (1973), *J. Biol. Chem. 248*, 2746.

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.

Hilborn, D. A., and Hammes, G. G. (1973), *Biochemistry 12*, 983.

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

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.

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

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

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.

Phillips, J. P., Freedman, L. D., and Craig, J. C. (1962), Organic Electronic Spectra Data, Vol. VI, New York, N. Y., Wiley-Interscience, p 61.

Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960), *J. Biol. Chem. 235*, 3322.

Racker, E., and Horstman, L. L. (1967), *J. Biol. Chem. 242*, 2547.