The role of tyrosine in the catalytic mechanism of nucleoside triphosphate hydrolysis by beef heart mitochondrial ATPase is explored. We compare the rates of the ATPase reaction by both nitrat ed and native F1 at both pH 8 and pH 6. The pH-activity profile of nitrated F1 is compared to the pH-activity profile of the unmodified enzyme. These data indicate that the phenolic group of an active-site tyrosine must be protonated during the hydrolysis reaction. Deuterium oxide is used in the reaction buffer to explore the role of protons in the ATPase reaction. Kinetic constants of the nucleoside triphosphates are obtained at various levels of D2O using both the nitrat ed and native forms of F1. Several nucleoside diphosphates are used as inhibitors of F1-catalyzed TTP hydrolysis. Dissociation constants of these inhibitors are obtained at both low and high concentrations of D2O for both the nitrated and native F1. We explore the possibility that a tyrosine and an arginine lie in close proximity in the F1 active site by studying the effects of sequential modification of arginine and tyrosine. These results are interpreted in terms of possible ATP hydrolysis mechanisms. Two possible roles for tyrosine in the hydrolysis of nucleoside triphosphates by F1 are suggested.

The F1 subunit of the heart mitochondrial ATPase has been extensively studied since it was first isolated in 1960 by Pullman et al. (1). One important aspect that has received attention in the last 10 years is the identification of the amino acids found in the active site and their roles in the catalytic mechanism. Senior (2) has shown that tyrosine could be present, and Ferguson et al. (3) have abhibited ATPase activity by modifying a single tyrosine with NBD-Cl. In addition, Esch and Allison (4, 5) have modified tyrosine residues with p-fluorosulfonylbenzoyl-5'-adenosine and have observed a decrease in F1 hydrolytic activity corresponding to an increase in the number of p-fluorosulfonylbenzoyl-5'-adenosine molecules bound. They also observed that modification by NBD-Cl did not affect the number of p-fluorosulfonylbenzoyl-5'-adenosine that could bind per F1 molecule and concluded that the two reagents reacted with different tyrosines. The presence of arginine has been demonstrated by Marcus et al. (6) who used 2,3-butanedione and phenylglyoxyxal to inhibit catalytic activity. Although work by Senior (2) and Godinot and colleagues (7) have shown that thiol modification does not affect ATPase activity, Pedersen (8) has suggested that a thiol is involved in anion binding.

The roles played by F1, active-site amino acid residues in the catalytic mechanism have not been determined. Arginine has been shown to participate in the active site of enzymes that act upon anionic substances (9-12), and it may play a similar role in F1, by binding to the phosphates of the nucleotide (6). The role of tyrosine, however, has not been explored. It has been shown by Ferguson et al. (3) that modification of a tyrosine residue in the active site by NBD-Cl does not affect the binding of aurovertin, yet the modification could be inhibited by the presence of substrate.

Tyrosine can also be modified using tetranitromethane to introduce a nitro group ortho to the phenolic group (13), lowering the pK of this group from about 10 to below 7 (14). The use of this reagent causes a loss of half of the ATPase activity (2), but whether this inhibition is due to the loss of an exchangeable proton is not clear. It is possible the additional nitro group per se is responsible for the decreased activity. One possible role for tyrosine is that of donor in a proton transfer reaction. It has been well established that proton transfer through the membrane accompanies ATP production, but, to date, no mechanism has been shown where proton transfer is directly coupled to ATP synthesis.

From studies relating either the $V_{max}$ or the $K_m$ for both the pH of the assay, Godinot et al. (15) determined the presence of four pK values essential to the activity of heart F1. These studies implicated the presence of an amino group in the F1 active site. Indeed, this was suggested earlier by Ferguson et al. (16) who showed that an NBD-Cl that was initially attached to an active-site tyrosine migrated to a nearby amino group. Another potentially important item observed by this group was that reaction of a tyrosine with NBD-Cl did not prevent nitration of another tyrosine in the active site (3).

In this communication we report the effect of tyrosyl nitration on the binding of nucleotides to F1, and the effect of nitration on the catalytic activity of F1. We describe the relationship of deuterium to the binding constants and catalytic velocities of the nucleotides with F1. Possible roles for tyrosine in the catalytic action are suggested.

**MATERIALS AND METHODS**

Beef heart mitochondrial ATPase (F1) was prepared according to the procedure of Spitzberg and Blair (17). The soluble enzyme was prepared fresh each day by centrifuging a small amount of the enzyme that was stored in 60% saturated (NH4)2SO4, and resuspending in a
solution containing 50 mM Tris at pH 8. Nitration was done in 2.0-ml aliquots containing 0.4-0.8 mg of enzyme and 0.02 ml of 0.1% tetranitromethane in 100 mM acetic acid and 50 mM triethanolamine hydrochloride; the final pH was adjusted with KOH or HCl. The assay temperature was 30 °C in all cases.

Because the coupled enzyme system described above could not be used to study nucleoside diphosphate inhibition of F1, a phosphate assay was used. These experiments were conducted in a total volume of 1.0 ml containing 5 mM ITP, 20 mM triethanolamine hydrochloride at pH 8, varying amounts of nucleotide inhibitor, and MgCl2 in 2 mM excess of the total nucleotide present. The temperature was maintained at 30 °C. The hydrolysis reaction was initiated by addition of F1 and terminated after 3 min by the addition of 0.5 ml of 10% sodium dodecyl sulfate. It was determined that, under the described conditions, production of phosphate by the F1-catalyzed ITP hydrolysis was linear with time. Appropriate zero-time controls were made by adding the sodium dodecyl sulfate to the mixture before the addition of enzyme. Analysis of the phosphate concentration was as described by Peterson (20). Because of the tendency for ITP to hydrolyze in all cases.

Due to deuterium ion affects a pH electrode differently than does hydrogen ion, it was necessary to treat the pH meter readings according to the equation of Salomon et al. (21). It was also determined that the use of deuterium in the assay media did not affect the coupled enzyme system or the phosphate determination reagents under our laboratory conditions.

Chemical modification studies were performed in order to examine the relation of the F1, active-site tyrosine to the active-site guani noid group. For all procedures the stored F1 was centrifuged and resuspended in 0.1 M borate buffer at pH 8. Modification of arginine by phenylglyoxal was done by the method of Marcus et al. (6), except that the protein was separated from unreacted phenylglyoxal and reaction products on a Sephadex G-25 column (25 × 1 cm) that had been equilibrated with 0.1 M borate buffer at pH 8. Fractions containing protein were located by monitoring the absorbance at 280 nm. These fractions were combined and the protein concentration determined by the Lowry procedure (18). A solution of unmodified F1 in 0.1 M borate buffer pH 8 was prepared so that its concentration was equal to that of the modified F1. One-ml aliquots of each protein solution were put in cuvettes and rapidly mixed with 5 μl of 20 mM NBD-Cl in ethanol. The reaction of NBD-Cl with the tyrosine was followed by monitoring the absorbance at 390 nm on a Perkin-Elmer Model 550 spectrophotometer.

Kinetics of Heart ATPase

It is generally accepted that the nucleoside diphosphates are competitive inhibitors of F1-catalyzed nucleoside triphosphate hydrolysis (22). Plots of 1/V versus inhibitor concentration will give slopes of (Km/Vmax SK) (23). Values for Km and Vmax for ITP hydrolysis in the different conditions (i.e. nitrotyrosine and/or at a specified concentration of D2O) were used to calculate Km from the slopes where the nucleoside diphosphates were used as inhibitors. Where the effects of both nitration and D2O were compared with the native enzyme in normal aqueous conditions, the Km and Vmax values for ITP hydrolysis were determined under the appropriate conditions prior to doing the kinetics using the nucleoside diphosphates as inhibitors.

The various buffers, tetranitromethane, and the nucleotides used were purchased from Sigma. Deuterium oxide was purchased from Aldrich. Other reagents used were of analytical purity obtained from commercial sources.

RESULTS

Tyrosine has been implicated in the catalytic mechanism of several enzymes (24-27). It has been shown to exist in the active site of the mitochondrial ATPase (2, 3), but its role has not been explored. Senior has shown that nitration of the active-site tyrosine decreased the rate of the ATP hydrolysis reaction (2). This could be caused by the ionization of the phenolic function of nitrotyrosine at pH 8. Alternatively, it could be that the added nitro group per se blocks substrate binding. To explore these possibilities, we compared velocities of F1-catalyzed nucleotide hydrolysis for both the nitrated and native forms of the enzyme. The hydrolysis experiments were performed both at pH 8 and at pH 6. At pH 6, both nitrated and native tyrosines are un-ionized. At pH 8 nitratred tyrosine is ionized while native tyrosine remains protonated. Substrate concentrations were varied and Vmax values were calculated from the y-intercepts of Lineweaver-Burk plots. From these experiments, ratios of hydrolysis rates were determined. These results are summarized in Table I. The ratios of the velocities of nucleotide hydrolysis by the native enzyme to the velocities of the nitrated enzyme at pH 8 show that the enzyme was indeed inhibited by the nitration of tyrosine. As can be seen from the ratio of the Vmax value at pH 8 to the Vmax value at pH 6 for the unmodified F1, velocities at pH 6 are slower than velocities at pH 8 before nitration. It is important to note that these ratios decrease upon nitration, demonstrating that at pH 6 the nitrated enzyme works relatively better than the native enzyme. It is also noteworthy that the same effect of pH and nitration were found at less than saturating levels of substrate with or without the presence of the activating bicarbonate ion. It must also be noted that, while the tyrosine pK decreases at least 3 units upon nitration (14), Vmax values for ATP hydrolysis at pH 8 only diminish by 60% (Table I). Although the data are not shown, there are about seven nitratred tyrosines/molecule of F1 in the cases used. It must also be emphasized that the effects seen upon nitration of F1 are similar when either ATP or ITP is the hydrolytic substrate. Since the kinetics of F1-catalyzed hydrolysis of these two nucleotides are different, it is essential to establish that the catalytic mechanism is the same in both cases. The data of Table I show that nitration of F1 causes a decrease in the Vmax for both ATP and ITP hydrolysis. It is interesting that for both nitrated and native F1, the pH dependence for ATP hydrolysis is steeper than for ITP hydrolysis (i.e. the velocity

<table>
<thead>
<tr>
<th>pH</th>
<th>Vmax (ATP)</th>
<th>Vmax (ITP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6</td>
<td>29.0</td>
<td>7.37</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>Vmax (ATP)</th>
<th>Vmax (ITP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6</td>
<td>4.25</td>
<td>1.90</td>
</tr>
</tbody>
</table>

| Native/nitrated, pH 8 | 2.84 | 9.59 | 14.2 | 30.1 |

| Native/nitrated, pH 6 | 0.412 | 2.48 | 2.17 | 4.01 |
Kinetics of Heart ATPase

ratio for pH 8 activity to pH 6 activity is greater for ATP than for ITP hydrolysis.

If the pH optimum of F₁ activity is dependent upon the degree of tyrosine ionization, the inhibition caused by nitration might be accompanied by a shift in the pH optimum. If tyrosine is involved in the catalytic mechanism, the lowered pK from 10 to 7 should cause whatever shift occurs to be in the downward direction. As can be seen in Fig. 1, nitration does cause a downward shift of the pH optimum for ATP hydrolysis.

In enzymes where nitration of an essential tyrosine reduces the catalytic activity by causing the phenolic function to be deprotonated, the activity can be at least partially restored by reducing the nitro group to an amine (14). This raises the pK from about 7 back to 10, reprotonating the tyrosine at most pH values. Table II compares the velocities of ATPase activity at saturating ATP levels before and after nitration and again after reduction of the nitrotyrosine with sodium dithionite. Nitration of F₁ caused a loss of 50% of the activity. Subsequent reduction of the nitro group to the amine restored the activity to about 90% of the rate of the unmodified F₁. This supports the hypothesis that the essential tyrosine must exist in a protonated state for maximal hydrolytic activity.

Table II

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>37.26</td>
<td>100.0</td>
</tr>
<tr>
<td>Nitrated</td>
<td>18.75</td>
<td>50.3</td>
</tr>
<tr>
<td>Nitrated, then reduced</td>
<td>34.80</td>
<td>93.4</td>
</tr>
</tbody>
</table>

The evidence from these experiments supports the hypothesis that tyrosine is not functioning directly in the catalytic mechanism as a proton donor but that ionization slows the overall reaction. In cases where a proton transfer is the rate-limiting step in an enzymatic mechanism, the presence of D₂O should inhibit the activity of the enzyme. Since ATP synthesis and hydrolysis is linked to transmembrane proton movement, the possible involvement of proton transfer in F₁ activity needs examination. Figs. 2 and 3 show the results of varying D₂O concentration at constant substrate concentrations. These experiments were done for both the native (Fig. 2) and nitrated (Fig. 3) F₁. With both nitrated and native F₁ at pH 8, the rate of ATP hydrolysis decreased as the concentration of D₂O increased. However, the rate of F₁-catalyzed ITP hydrolysis at pH 8 (with less than saturating substrate) remained unchanged for native F₁ and actually increased with nitrated F₁. At pH 6, the F₁-catalyzed hydrolysis of ATP and ITP decreased with increasing D₂O concentration whether F₁ was nitrated or not.

Further experiments were done to determine what effects nitration and D₂O would have on the Km and Vmax values for the nucleoside triphosphate hydrolysis by F₁. From the data of Table III it can be seen that, regardless of the F₁ substrate, nitration had very little affect on Km values. Generally, the effect of D₂O was to raise the Km values for the native F₁ and to lower the Km values for the nitrated F₁. These trends seemed to hold regardless of the F₁ substrate: ATP, ITP, εATP, or εCTP. Although the data are not shown, the same effects were seen when CTP and UTP were the F₁ substrate.

Table IV shows the effect of nitration at varying D₂O levels on the Vmax values for six different substrates. Several results must be noted. First, at any D₂O concentration, nitration decreases the Vmax value obtained. Second, the ATP hydrolysis Vmax value decreases in the nitrated or native F₁ as a function of increasing D₂O concentration. Third, for the native F₁, the Vmax value for ITP and εATP hydrolysis in
creases with increasing D$_2$O. Finally, for the nitrated F$_1$, the ITP and eATP hydrolysis $V_{\text{max}}$ values also increase with increasing D$_2$O, although for ITP hydrolysis, the change is slight. It should be emphasized that the data of Table IV are $V_{\text{max}}$ values for the respective experimental conditions, while the data of Figs. 2 and 3 were obtained with high, but not necessarily saturating, substrate concentrations.

Because it was apparent that the tyrosine was not directly involved in the catalytic mechanism, it seemed possible that it might interact with the base portion of the nucleotide. Nitration had essentially no effect on the $K_m$ values of the purines tested (including eCTP, which strongly resembles a purine) so that it seemed possible that any interaction between the tyrosine and the base could occur at the N7 of the 5-membered ring. Because the pyrimidines lack this smaller ring, we tested the effects of nitration on CTP hydrolysis. The effects of nitration were to increase dramatically the $K_m$ value (see Table III) and double the $V_{\text{max}}$ value (see Table IV). To determine whether these phenomena were characteristic of all pyrimidine substrates, we tested the effects that nitration of F$_1$ has on UTP hydrolysis. As can be seen in Tables III and IV, nitration caused the $K_m$ value to double and the $V_{\text{max}}$ value to decrease.

We have previously observed that hexokinase will not use CTP as a substrate but will use eCTP (28). Since F$_1$ is also a phosphoryl transfer enzyme, it was of interest to determine if the F$_1$ activity was similarly affected by substrate size. Fig. 4 compares the F$_1$-catalyzed hydrolysis of CTP with the F$_1$-catalyzed hydrolysis of its analog, eCTP. As can be seen, there is a marked increase in the ability for F$_1$ to hydrolyze eCTP relative to its ability to hydrolyze CTP.

Other experiments were performed to determine what ef-

<table>
<thead>
<tr>
<th>% D$_2$O</th>
<th>ATP</th>
<th>ITP</th>
<th>eATP</th>
<th>eCTP</th>
<th>UTP</th>
<th>CTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.209</td>
<td>0.256</td>
<td>1.74</td>
<td>1.86</td>
<td>0.535</td>
<td>0.500</td>
</tr>
<tr>
<td>20</td>
<td>0.209</td>
<td>0.256</td>
<td>1.74</td>
<td>1.86</td>
<td>0.535</td>
<td>0.500</td>
</tr>
<tr>
<td>40</td>
<td>0.209</td>
<td>0.256</td>
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<td>1.86</td>
<td>0.535</td>
<td>0.500</td>
</tr>
<tr>
<td>60</td>
<td>0.209</td>
<td>0.256</td>
<td>1.74</td>
<td>1.86</td>
<td>0.535</td>
<td>0.500</td>
</tr>
<tr>
<td>80</td>
<td>0.209</td>
<td>0.256</td>
<td>1.74</td>
<td>1.86</td>
<td>0.535</td>
<td>0.500</td>
</tr>
</tbody>
</table>

*Native F$_1$.

*Nitrated F$_1$.
Table V
Effects of D2O and nitrination on nucleoside diphosphate inhibition of ITP hydrolysis by F1.

<table>
<thead>
<tr>
<th>F1</th>
<th>% D2O</th>
<th>ADP K</th>
<th>IDP K</th>
<th>tADP  K</th>
<th>2-tADP  K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0</td>
<td>0.00855</td>
<td>1.58</td>
<td>0.376</td>
<td>0.0178</td>
</tr>
<tr>
<td>80</td>
<td>0.00843</td>
<td>0.612</td>
<td>0.181</td>
<td>0.0071</td>
<td></td>
</tr>
<tr>
<td>Nitrated</td>
<td>0</td>
<td>0.0341</td>
<td>2.37</td>
<td>1.06</td>
<td>0.0323</td>
</tr>
<tr>
<td>80</td>
<td>0.00799</td>
<td>0.760</td>
<td>0.456</td>
<td>0.0108</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. Modification of tyrosyl residues in F1, as a function of time using unmodified F1 (—), and F1 modified by phenylglyoxal (—). Conditions are as described under “Materials and Methods.”

The observation of the shift in the pH optimum shown in Fig. 1 supports the hypothesis that the F1 active-site tyrosine must be capable of protonation. It is interesting to compare suggested that the guanido group of an F1 active-site arginine is also involved in nucleotide binding. To examine the possible relation of the relative guanido group with the tyrosine(s) of the active site, a multiple modification experiment was performed. This experiment involved the covalent modification of the F1 active-site arginine with phenylglyoxal (6) followed by active-site tyrosine modification with NBD-Cl (16). The binding of the NBD-Cl to the F1 inactive-site tyrosine is accompanied by an absorbance change at 390 nm that can be conveniently monitored. The results of such an experiment are shown in Fig. 5. As can be seen, the NBD-Cl binding to F1 is significantly slower on the phenylglyoxal-modified F1, as compared to the unmodified control.

DISCUSSION

The phenolic group of tyrosine can exist in either a protonated or unprotonated state, depending upon its environment. Although the tyrosine phenolic pK is about 10 in aqueous solution, local perturbations in the environment could change its ionization potential. Since a tyrosine appears to be important to the catalytic function of F1 (2, 3), a knowledge of its ionic state can provide information regarding the role it plays in nucleoside triphosphate hydrolysis. Our data indicate that the tyrosine must be protonated or at least be capable of protonation for the enzyme to work efficiently. Nitration of tyrosine lowers the pK of the hydroxyl to about 7 (14). This is accompanied by a loss in the observed hydrolysis rate (2, 3) (see also Table I at pH 8 where it is normally assayed. At this pH, the nitrated tyrosine is about 90% deprotonated, while native tyrosine is nearly completely protonated.

Reduction of the nitro group to an amine raises the pK back to about 10, effectively restoring the tyrosine to a protonated state at pH 8. It is also possible that introduction of the nitro group could cause steric hindrance for binding or catalysis. If the loss of activity is due to steric hindrance rather than the change in ionic state of the tyrosyl residue, then reduction of the nitro group to the amine would have little effect toward restoring the activity of the enzyme. As can be seen in Table II, the effect of reduction is the recovery of nearly all the activity lost when F1 was nitrated. Hence, it appears that the loss of activity is due to the lower pK of the phenolic group rather than to a steric effect from the presence of the nitro group.

If F1 is assayed at a pH where the nitrated tyrosine is protonated, it should function more like the unmodified enzyme does at pH 8. Table I shows that assaying the nitrated F1-catalyzed hydrolysis of both ATP and ITP at pH 6 does improve the relative activity. If protonation of the tyrosine is required for activity of F1, there should be a greater percentage of the activity lost by the native enzyme when the assay pH is changed from 8 to 6 than would be lost by the nitrated enzyme. As can be seen in Table I, the ratios of pH 8 activity to pH 6 activity is larger for the native enzyme than for the nitrated F1. That this effect occurs for the hydrolyses of both ATP and ITP demonstrates that the effect of nitration is at some area of the active site that is binding to the substrate in a region common to both ATP and ITP. It is interesting to note, too, that the same effect was observed at both high and low substrate concentrations. In addition, the same effect was observed whether the activating ion bicarbonate was present or absent. This indicates that the effect of nitration is felt more in the nucleotide locale than in the anion binding site where a potentially oxidizable sulfhydryl group has been implicated (30).

The observation of the shift in the pH optimum shown in Fig. 1 supports the hypothesis that the F1 active-site tyrosine must be capable of protonation. It is interesting to compare...
the pK values derived from the profiles in Fig. 1 with those obtained by Godinot et al. (15) from pig heart mitochondria. From a similar plot, they obtained pK values of 6.1 and 7.8, and from a plot of $V_{max}/K_M$, they obtained another pK value of 7.2. The profile of our native F₁ yields pK values of 6.4 and 7.4, while the profile of the nitrated enzyme produces pK values of 6.1 and 7.2. Whether our values differ from theirs because of a difference in the source of enzyme or in its preparation is not clear. In addition, the decrease in the two pK values from the native to the nitrated enzyme may indicate a change in the active-site environment caused by a slight conformational change.

It seems clear from our data that the $K_m$ values of purine riboside triphosphate hydrolysis by F₁ are not merely dissociation constants. While nitration did not particularly affect the $K_m$ values of the purine riboside triphosphates tested (see Table III), it caused appreciable increases in the $K_m$ values of the corresponding nucleoside diphosphates (see Table V). This could imply that product release is not rate-limiting or that the dissociation constants measured may result from nucleoside diphosphate interaction at another site, perhaps the regulatory site postulated by Hilborn and Hammes (31) and later by us (32).

That the F₁ activity is strongly influenced by the size of the nucleotide as well as the location of important nitrogens in the rings is clear from the use of CTP as a hydrolytic substrate. The low activity of the pyrimidine riboside triphosphates and the extremely poor capability of their corresponding diphosphates to act as inhibitors of nucleoside triphosphate hydrolysis are well known (32). When the etheno function is added to the pyrimidine CTP, the rate of F₁-catalyzed hydrolysis dramatically increases (see Fig. 4) and the $K_m$ value becomes much lower (see Table III). The $K_m$ value for the interaction of a substrate with an enzyme is defined as $(k_+ + k_-)k_i$, where $k_-$ and $k_i$ are the rate constants for the dissociation and formation of the central complex, respectively, and $k_i$ is the rate constant for the formation of free enzyme and product from the central complex. The maximal rate of eCTP hydrolysis by F₁ is much greater than the maximal rate of CTP hydrolysis by F₁ (see Table IV), indicating that the $k_i$ is greater when eCTP is the F₁ substrate. Therefore, the decrease in the $K_m$ value when the etheno function is added to CTP is primarily due to enhanced binding. This implies that the etheno nucleotide binds more strongly to the enzyme than does CTP. Although eCDP was not used as an inhibitor of the hydrolysis reaction, its similarity to eCTP suggests that it would be a better inhibitor than its parent compound, CDP. Another observation supports the hypothesis that the size of the nucleotide is important. While the $K_m$ values of the pyrimidine riboside triphosphates were not appreciably altered upon nitration of the tyrosine, the $K_m$ values of the pyrimidine riboside triphosphates significantly increased. It may be that the pyrimidine compounds are incapable of producing the conformational change necessary in the enzyme to effect proper catalysis. On the other hand, the substrate binding site may be too large for the smaller pyrimidine nucleotides to bind tightly.

Another interesting and unexpected observation of the effects of nitration F₁ is the increase in the $V_{max}$ value for CTP hydrolysis. Since nitration of F₁ does not exhibit the same characteristic towards UTP, it is probable that the amino group in the 6 position plays a specialized role that is not presently understood.

The tyrosine in the active-site of F₁ may bind either to the sugar or to the base of the nucleotide. If it binds to the sugar, it must be through a hydrogen bond to one of the oxygens. This seems unlikely since nitration of the enzyme and the presence of deuterium in the assay medium had the same effects upon the dissociation constant for Z-DADP from F₁ as they had on the $K_m$ values for the other nucleotide inhibitors (see Table V). Likewise, since the $K_m$ values for all nucleoside diphosphates were similarly affected by nitration of the enzyme or by the presence of deuterium, the tyrosine cannot be binding to an amino group or an oxygen of a hydroxy group attached to the ring system of the base. This is further supported by the similarity of the trends observed in the $K_m$ values for the nucleotides upon nitration of the enzyme or the presence of deuterium. However, Inoue et al. (33) have shown that at least two nitrogens of ethenoadenosine do protonate. One of these is the nitrogen opposite the glycosidic bond in the five-membered ring common to all purines; another is the nitrogen in the etheno ring that was originally the free amino group in ATP. The two nitrogens in the six-membered ring of non-ethenopurines are similar to the nitrogen in the etheno ring and should be capable of accepting a proton from tyrosine to form a hydrogen bond. Nitration would remove the hydrogen from the tyrosine, decreasing the ability to bind. This is reflected in the higher $K_m$ values (see Table V) of the nucleoside diphosphates when F₁ is nitrated. However, this could be questioned as nitration causes a drop in the $K_m$ values of the nucleoside triphosphates at high D₂O levels (see Table III). But, as mentioned above, the $K_m$ value for nucleoside triphosphate hydrolysis appears to include a catalytic rate term and this may be reflected in the lower $K_m$ values upon nitration. If tyrosine is binding to one of the nitrogens in the ring system, deuterium would strengthen the hydrogen bond, lowering the dissociation constants. As seen in Table V, an increase in D₂O concentration causes a decrease in the $K_m$ values of the nucleoside diphosphates. A similar trend is seen for the $K_m$ values of the nucleoside triphosphates (see Table III), but these are more difficult to interpret.

A second possible role for tyrosine in nucleoside triphosphate catalysis involves interaction with a nearby arginine residue. Ferguson et al. (16) demonstrated that there is an amino group near the tyrosine modified with NBD-CI to which the ligand migrates irreversibly. When Marcus et al. (6) determined the presence of arginine in the active-site of F₁, they noticed that incorporation of one phenylglyoxal/ enzyme molecule caused 50% inactivation. They interpreted this to mean that there was likely one or two active sites. However, no attempt was made to determine the number of arginines/active site. In addition, the presence of more than one tyrosine residue was implicated by the work of Ferguson et al. (3) and Esch and Allison (6). Both groups found that the interaction of an NBD-CI with an essential tyrosine did not interfere with the subsequent addition of a second selective tyrosyl-modifying reagent with another tyrosine in the active site. It could be that there are two separate tyrosines with different properties in or near the active site. In experiments designed to determine whether modification of active-site tyrosine affected subsequent arginylation modification, Steinmeier and Wang (34) found that the NBD-CI-tyrosine interaction was not affected by the modification of arginine with butanedione. They interpreted this to mean that there was no direct competition between the two labels. However, our data, as shown in Fig. 5, indicate that modification of arginine with phenylglyoxal inhibits the subsequent tyrosyl modification by NBD-CI. When we reacted F₁ with phenylglyoxal at an active-site guanido group, this large chemical modification prevented the NBD-CI-tyrosine interaction. The difference in our results compared to those reported previously (34) could be due to the order of modification or the size of the guanido-binding compound. Nonetheless, it appears that the active-site guanido group could be close to the tyrosine being modified by
NBD-Cl. A possible role for the active-site tyrosine could be to control the protonation of the nearby guanido group.

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34. Steinmeier, R. C., and Wang, J. H. (1979) Biochemistry 18, 11-18