Phosphorus-31 Nuclear Magnetic Resonance Application to Positional Isotope Exchange Reactions Catalyzed by *Escherichia coli* Carbamoyl-Phosphate Synthetase: Analysis of Forward and Reverse Enzymatic Reactions†

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ABSTRACT: ^{31}P NMR was used to follow the positional isotope exchange reactions catalyzed by carbamoyl-phosphate synthetase from *Escherichia coli*. In agreement with the data of Wimmer et al. [Wimmer, M. J., Rose, I. A., Powers, S. G., & Meister, A. (1979) *J. Biol. Chem. 254*, 1854] carbamoyl-phosphate synthetase was shown to catalyze the $\beta\gamma$-bridge:β-nonbridge positional oxygen exchange in [γ-^{18}O]ATP. The ratio of micromoles of ATP exchanged to micromoles of ADP produced was 0.42–0.46 in the presence or absence of L-ornithine. There was no detectable enzyme-catalyzed exchange in the presence of L-glutamine which is consistent with the previously published steady-state kinetic mechanism [Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1978) *Biochemistry 17*, 5587]. These positional isotope-exchange data along with our rapid-quench data [Raushel, F. M., & Villafranca, J. J. (1979) *Biochemistry 18*, 3424] permit us to calculate the rate constants for the partitioning of intermediates in the reaction. The scheme is

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \xrightarrow{k_5} E + P$$

where EA is the enzyme–MgATP–HCO_3^- Michaelis complex and EP is the enzyme–MgADP–carboxy phosphate complex, and the values for k_3, k_4, and k_5 are 4.2 s^{-1}, 0.10 s^{-1}, and 0.21 s^{-1}, respectively. In the partial back-reaction of carbamoyl-phosphate synthetase, the enzyme was shown to catalyze the bridge:nonbridge oxygen exchange in ^{18}O-labeled carbamoyl phosphate in the presence of MgADP. The rate of exchange was 4 times faster than the net synthesis of ATP. This exchange reaction is consistent with the intermediate formation of carbamate. There was no detectable exchange in the absence of MgADP. Overall, these data support the formation of two intermediates, viz., carboxy phosphate and carbamate, in the overall reaction catalyzed by carbamoyl-phosphate synthetase. Both intermediates are formed faster than or equal to the fastest step in the reaction.

Carbamoyl-phosphate synthetase from *Escherichia coli* catalyzes the reaction

$$2MgATP + HCO_3^- + \text{glutamine} \rightarrow 2MgADP + P_i + \text{glutamate} + \text{carbamoyl-P} \quad (1)$$

In addition to the overall reaction the enzyme also catalyzes a bicarbonate-dependent ATPase reaction and the synthesis of ATP from ADP and carbamoyl phosphate (Anderson & Meister, 1966):

$$MgATP \xrightarrow{HCO_3^-} MgADP + P_i \quad (2)$$

$$MgADP + \text{carbamoyl-P} \rightarrow MgATP + HCO_3^- + NH_3 \quad (3)$$

Anderson & Meister (1965) have proposed that the enzyme catalyzes the overall reaction via the steps

$$MgATP + HCO_3^- \rightarrow MgADP + {}^-O_2COPO_3^{2-} \quad (4)$$

$${}^-O_2COPO_3^{2-} + NH_3 \rightarrow NH_2CO_2^- + P_i \quad (5)$$

$$NH_2CO_2^- + MgATP \rightarrow NH_2COPO_3^{2-} + MgADP \quad (6)$$

Thus, there are two postulated intermediates in the reaction sequence, carboxy phosphate and carbamate. In this paper we describe experiments designed to test for (1) the existence of these intermediates and (2) their kinetic formation and breakdown during catalysis.

Recently, Wimmer et al. (1979) have provided evidence that carboxyphosphate is formed in the enzymatic reaction fast enough to be considered a kinetically competent intermediate in catalysis. In their experiment the positional isotope exchange reaction catalyzed during the ATPase reaction was followed. The positional isotope exchange technique was developed by Midelfort & Rose (1976) to follow the positional exchange of ^{18}O in ATP from a bridge (β,γ) to a nonbridge position (β). In the first application of the technique, they showed that γ-glutamyl phosphate was an intermediate formed during the glutamine synthetase reaction. Wimmer et al. (1979) demonstrated that carbamoyl-phosphate synthetase catalyzed a HCO_3^--dependent exchange of ^{18}O in the reaction in Scheme I. The exchange is due to the reversible formation of enzyme-bound ADP and carboxy phosphate. Because of rotational equivalence of the three β-nonbridge oxygens of ADP, the reformation of ATP results in a 67% probability that the labeled oxygen will be found in one of the β-nonbridge positions of ATP. The detection of this exchange is made possible by an extensive series of enzymatic degradations of the ATP, followed by mass spectral analysis of the products (Midelfort & Rose, 1976).

Cohn & Hu (1978) have recently developed an NMR method for determining the distribution of ^{18}O atoms bonded to phosphorus. They discovered that the replacement of ^{16}O by ^{18}O results in about a 0.02-ppm upfield chemical shift for

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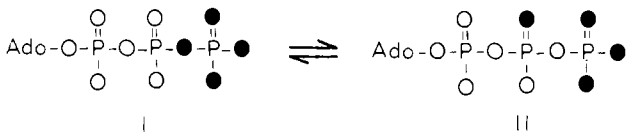
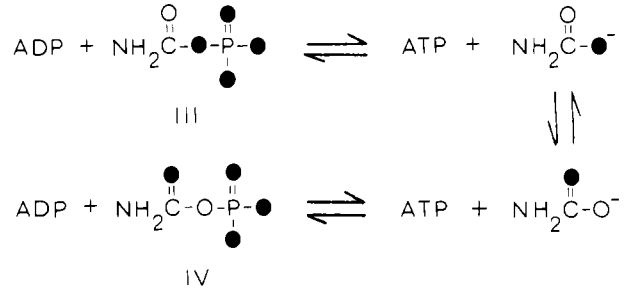
Scheme I



Scheme II



each substituted oxygen. This technique has been used predominantly in the measurement of enzyme-catalyzed oxygen exchange from phosphate esters and P_i with water [Cohn & Rao, 1979; Villafranca & Raushel (1980) and references cited therein].

In this report we have applied this NMR technique to monitor the positional isotope exchange reaction catalyzed by carbamoyl-phosphate synthetase in the hydrolysis of ATP and in the partial reverse reaction. The data for the partial reverse reaction could be used to detect the formation of carbamate, the other postulated intermediate. If carbamate was an intermediate, then the exchange depicted in Scheme II would be possible. The exchange could be followed by NMR because the new product (IV) will have only three ^{18}O atoms bonded to the phosphate rather than the original four. ^{31}P NMR experiments in this paper describe the measurements of the positional isotope exchange for the ATPase reaction under a variety of conditions and the presence of a positional isotope exchange in carbamoyl phosphate catalyzed in the partial back-reaction.

Materials and Methods

Carbamoyl-phosphate synthetase was isolated from *E. coli* according to the procedure of Matthews & Anderson (1972). $[^{18}O]KH_2PO_4$ was synthesized from PCl_5 and $H_2{}^{18}O$ according to the procedure of Risley & Van Etten (1978). NMR analysis (Cohn & Hu, 1978) showed that the sample contained 95% $[^{18}O_4]KH_2PO_4$ and 5% $[^{18}O_3,{}^{16}O]KH_2PO_4$. Ornithine transcarbamylase from *Streptococcus faecalis* was a gift from Dr. Margaret Marshall.

Preparation of $[\gamma\text{-}^{18}O]ATP$ *(I).* ^{18}O-Labeled ATP (I) was synthesized from ADP-morpholidate and $[^{18}O_4]P_i$ according to the procedure of Wehrli et al. (1965) as modified by Midelfort & Rose (1976). The yield from 500 μmol of ADP-morpholidate and 1.5 mmol of $[^{18}O_4]P_i$ was 50%. NMR analysis (Cohn & Hu, 1978) showed that the distribution of species containing four and three ^{18}O atoms in the γ-PO_4 was 80 and 20%, respectively.

Preparation of Carbamoyl $[^{18}O]$*Phosphate (III).* Carbamoyl phosphate enriched with ^{18}O was made according to the procedure of Mokrasch et al. (1960). In a 10-mm NMR tube 0.05 mL of 1 M KOH, 0.05 mL of 1 M potassium acetate, pH 4.96, 0.15 mL of 1 M KOCN, and 7.0 mg of $[^{18}O_4]K$-H_2PO_4 were incubated for 15 min at 35 °C. Another 0.10 mL of 1 M KOCN was added and the mixture was incubated for 15 min. The sample was then cooled and then diluted to 2.0 mL to prepare, in final concentration, 180 mM KCl, 90 mM Hepes, pH 7.5, 20 mM Mg^{2+}, 10 mM L-ornithine, 100 mM glucose, 25 units of yeast hexokinase, 4.5 mM EDTA, 20% D_2O, and various amounts of ADP. Integration of the ^{31}P NMR spectrum showed that the sample was 8 mM in carbamoyl phosphate and 16 mM in P_i.

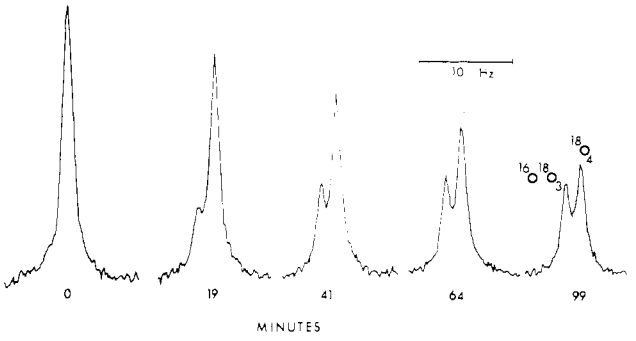


FIGURE 1: ^{31}P NMR spectra at 81.01 MHz of carbamoyl-phosphate synthesized from $[^{18}O_4]P_i$ after incubation for the indicated times with carbamoyl-phosphate synthetase and MgADP. In each spectrum the most upfield peak is due to phosphorus atom of carbamoyl-phosphate in which the bridge oxygen is labeled with ^{18}O (structure III). The peak at lower field is due to carbamoyl phosphate in which the carbonyl oxygen is labeled with ^{18}O (structure IV).

The positional isotope exchange in carbamoyl phosphate was measured at 25 °C. The reaction was initiated by adding 1 mg of carbamoyl-phosphate synthetase to the solution described above. A ^{31}P NMR spectrum was obtained at various intervals by using a Brüker WP-200 NMR spectrometer operating at 81.01 MHz. The sweep width in these experiments was 1000 Hz, and 40 scans were accumulated with an aquisition time of 8 s.

Positional Isotope Exchange in $[\gamma\text{-}^{18}O]ATP$. Carbamoyl-phosphate synthetase (0.06–0.6 mg) was incubated with 67 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 133 mM KCl, 13% D_2O, 10 mM HCO_3^-, 7.33 mM $[\gamma\text{-}^{18}O]$ATP, 10 mM ornithine, and, when included, 10 mM glutamine, 34 mM $(NH_4)_2SO_4$, and 150 units of ornithine transcarbamylase in a volume of 1.5 mL. After the chemical reaction had proceeded to ~50% completion, the reaction was stopped by the addition of a few drops of CCl_4 and 40 mM EDTA. The solution was vigorously vortexed and centrifuged, and the pH was adjusted to 9.2. The ^{31}P NMR spectrum was taken at 81.01 MHz as described above by using a sweep width of 2000 Hz. A total of 2500 scans were accumulated with an acquisition time of 3.7 s.

Results

Positional Isotope Exchange of the Partial Reverse Reaction. Shown in Figure 1 is a series of NMR spectra of ^{18}O-enriched carbamoyl phosphate (III) in the presence of 9.0 mM MgADP taken at various times after the addition of carbamoyl-phosphate synthetase. Hexokinase and glucose were added to the reaction mixture to prevent any reversal of the net chemical reaction. The figure clearly shows that the enzyme catalyzes the bridge to nonbridge exchange as depicted in Scheme II. At equilibrium both species (III and IV) will be of equal population.

A complete set of data gathered under the conditions described above and under Materials and Methods is given in Figure 2. The data in Figure 2 are presented as the percentage of completion of the chemical and exchange reactions. Analysis of the data according to the equations derived by Litwin & Wimmer (1979) shows that the exchange rate is 3.9 ± 0.5 times faster than the net chemical reaction, and the solid line drawn through the open circles in Figure 2 is drawn for this ratio.

Table I: Positional Isotope Exchange of [γ-^{18}O]ATP Catalyzed by Carbamoyl-Phosphate Synthetase[a]

expt	additions	enzyme (mg)	minutes	fraction of chemical reaction	percentage of γ-[^{16}O,^{18}O$_3$]-P	fraction of exchange reaction	μmol of ATP exch./ μmol of ADP prod.
A	none	0			20		
B	HCO_3^-	0.60	180	0.50	33	0.27	0.46
C	HCO_3^- and glutamine	0.06	330	0.65	23	0.06	0.06
D	HCO_3^-, glutamine, $(NH_4)_2SO_4$, and ornithine transcarbamylase	0.06	120	0.55	20	0	

[a] Reaction conditions: 67 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 133 mM KCl, 10 mM ornithine, 13% D_2O, 10 mM HCO_3^-, 7.33 mM [γ-^{18}O]ATP, and, when included, 10 mM glutamine, 34 mM $(NH_4)_2SO_4$, and 150 units of ornithine transcarbamylase. Only half of the doublet of the γ-P of the [γ-^{18}O]ATP was measured since the other half was obscured by the β-P of the ADP produced during the reaction.

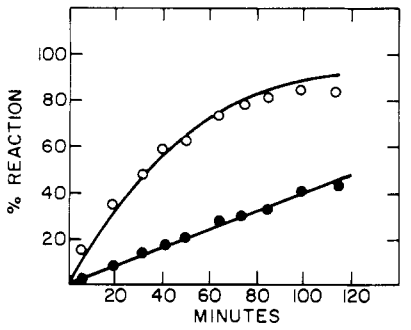


FIGURE 2: Plot of the chemical reaction (●) and the positional isotope exchange reaction (○) of the partial reverse reaction vs. time. The data were taken from NMR spectra as in Figure 1. The positional isotope exchange reaction was monitored by following the distribution of the $^{18}O_4$ and $^{18}O_3$,^{16}O peaks. The chemical reaction rate was monitored by following the decrease in the integrated areas of the carbamoyl phosphate resonances. Additional details are given in the text.

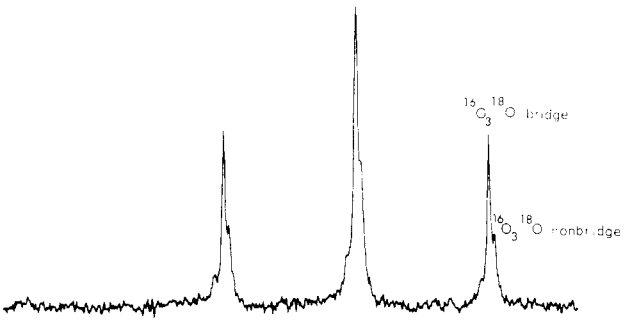


FIGURE 3: ^{31}P NMR spectra of the β-P of [γ-^{18}O]ATP before and after incubation with carbamoyl-phosphate synthetase and HCO_3^-. The small peaks to higher magnetic field are due to the larger chemical shift effect by ^{18}O in the β-nonbridge position than in the $\beta\gamma$-bridge position. Additional details are given in the text and in Table I.

Similar data were obtained when the ADP concentration was lowered to 1.0 mM. The ratio of the exchange rate to the chemical rate was 4.1 ± 0.4 for this experiment. In the absence of MgADP there was no detectable exchange after 2.0 h.

Positional Isotope Exchange in the ATPase Reaction. Table I presents data from a series of ^{31}P NMR experiments showing the positional isotope exchange in the $\beta\gamma$ bridge of [γ-^{18}O]ATP during the ATPase reaction under a variety of conditions. Experiment A presents the percentage of the γ-[^{16}O,^{18}O$_3$]P species in [γ-^{18}O]ATP before the addition of enzyme. After the addition of enzyme, the ^{31}P NMR spectrum was recorded after 50% of the ATP had been hydrolyzed and the percentage of γ-[^{16}O,^{18}O$_3$]P species was calculated. The ratio of micromoles of [γ-^{18}O]ATP exchanged to micromoles of ADP produced is 0.46 (Table I). Experiment C presents the result upon adding 10 mM glutamine to the reaction mixture. As expected, the ratio is reduced because at saturating glutamine the chemical reaction rate is increased ~10-fold (Anderson & Meister, 1966). The ratio of micromoles of [γ-^{18}O]ATP exchanged to micromoles of ADP produced is 0.06 (Table I) with glutamine present.

When glutamine is added to the reaction mixture, carbamoyl phosphate is produced. The carbamoyl phosphate must be enzymatically removed to prevent any reversal of the net chemical reaction since the products ADP and carbamoyl phosphate can react to generate ATP. This partial back-reaction rate is ~15% of the synthetase reaction rate. In experiment D (Table I) ornithine transcarbamylase was added to remove carbamoyl phosphate. Additionally, $(NH_4)_2SO_4$ was added to saturate the ammonia sites on those enzyme molecules that could possibly have inactive glutamine sites. There was no detectable (<5%) positional isotope exchange in the presence of glutamine, NH_4^+, and ornithine transcarbamylase. All of the data are summarized in Table I.

Shown in Figure 3 is a spectrum of the β-P of the [γ-^{18}O]ATP isolated from reaction mixture B (Table I). The β-P of ATP clearly shows a peak at 0.01 ppm upfield from the main peaks. This is attributed to the larger upfield shift for the ^{18}O in the β-nonbridge position than for the $\beta\gamma$-bridge position. Cohn & Hu (1980) have found the difference to be 0.012 ppm.

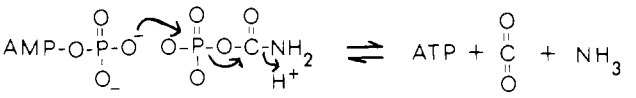
The ratio of the positional isotope exchange rate to the chemical rate at 37 °C and in the absence of L-ornithine was found to be 0.42.

Discussion

Back-reaction. In the partial reverse reaction catalyzed by *E. coli* carbamoyl-phosphate synthetase, the positional isotope exchange reaction rate was found to be 4 times faster than the steady-state rate of ATP synthesis. Thus, the chemical reaction rate cannot be limited by a bond breaking step because the positional isotope exchange rate is the minimal possible rate for bond cleavage. The most likely enzyme-bound intermediate for this exchange reaction is carbamate as depicted in Scheme II. In this scheme ADP and carbamoyl phosphate react to form enzyme-bound ATP and carbamate. Due to the rotational equivalence of the oxygens of carbamate, the labeled oxygen can occupy either position when carbamoyl phosphate is resynthesized on the enzyme. This exchange reaction is readily detected by ^{31}P NMR because the new positional isomer of carbamoyl phosphate will have only three atoms of ^{18}O bonded to phosphorus instead of the original four. This reaction is clearly seen in Figure 1.

The complete reverse reaction of carbamoyl-phosphate synthetase has not been demonstrated because the rate of incorporation of $^{32}P_i$ into ATP occurs at a rate of less than 1% of the rate of formation of ATP from ADP and carbamoyl phosphate in the first step of the back-reaction (Raushel &

Scheme III



Villafranca, 1979). Therefore, some intermediate in the reaction sequence must be irreversibly dissociating from the enzyme surface much faster than its reaction with P_i. If this intermediate is carbamate, then the release of carbamate from the enzyme probably limits the rate of the partial back-reaction. The positional isotope exchange ratio of 4 also demonstrates that the proposed enzyme–carbamate–ATP complex partitions back to ADP and carbamoyl phosphate (in solution) 4 times faster than the release of carbamate and ATP into solution.

Alternatively, carbamate may not be the intermediate. However, this would require that ADP and carbamoyl phosphate react in a concerted mechanism to directly form CO_2, NH_3, and ATP as depicted in Scheme III. This would also require that the reaction is freely reversible on the enzyme and that CO_2 is free to rotate. Interestingly, Jones (1976) has failed to detect any exchange of $^{15}NH_4^+$ into carbamoyl phosphate during conditions of the partial back-reaction with carbamoyl-phosphate synthetase from frog liver. However, this result does not disprove a concerted mechanism since NH_3 may not exchange from the enzyme–ATP–CO_2–NH_3 complex. At present, all the data are best fit by invoking the formation of enzyme-bound carbamate from ADP and carbamoyl phosphate with free rotation of the carboxyl of carbamate.

There was also no detectable positional isotope exchange in the absence of added ADP. This result makes the possibility of formation of covalent phosphoenzyme or the likelihood of a metaphosphate intermediate unlikely unless rotation of the bound carboxyl is hindered. Formation of a metaphosphate intermediate has been proposed for pyruvate kinase (Lowe & Sproat, 1978), but several other possible interpretations have not been ruled out.

ATPase Reaction. In the bicarbonate-dependent hydrolysis of ATP by carbamoyl-phosphate synthetase, the positional isotope exchange reaction rate is 0.46 times as fast as the rate of formation of ADP in the presence of the allosteric activator, L-ornithine.

We have previously shown that ATP dissociates from the enzyme very rapidly compared with the steady-state rate (k_{cat}) of the ATPase reaction in the presence of ornithine (Raushel & Villafranca, 1979). Therefore, the rate of release of ATP does not affect the positional isotope exchange ratio in the presence of ornithine. Since ornithine has been shown to only decrease the K_m for ATP (Anderson & Marvin, 1968), the rate constant for the release of ATP should be even faster in the absence of ornithine. Therefore, the addition of ornithine to the reaction mixture should not change the positional isotope exchange ratio. This is observed experimentally.

In the absence of ornithine and at 37 °C, Wimmer et al. (1979) have determined the positional isotope exchange ratio to be 1.4–1.7 using a mass spectral analysis of [γ-^{18}O]ATP. We have found the ratio to be 0.42 under their conditions. The reason for the difference is not clear. However, both sets of experimental data clearly show significant enzyme-catalyzed positional isotope exchange.

The ratio of the exchange rate to the chemical rate was diminished to 0.06 in the presence of glutamine. This is to be expected because the synthesis of carbamoyl phosphate in the presence of glutamine is at least 10-fold faster than the ATPase rate and the concentration of the E–ADP–carboxy phosphate complex must surely be decreased in the presence of saturating amounts of glutamine. The ratio, however, did not go to zero (Table I) as would be expected if the mechanism were ordered and there was no additional exchange from the second ATP site. An explanation of this observation follows. In the ATPase reaction there is no reversal of the reaction from ADP and P_i. However, when glutamine is in the reaction mixture, carbamoyl phosphate is produced. The maximal rate of ATP synthesis from ADP and carbamoyl phosphate is faster than the ATPase rate and ~15% of the overall carbamoyl phosphate synthesis rate. Therefore, substantial exchange can occur by simple partial reversal of the forward reaction by the components produced in solution. This would lead to false high rates for the exchange reactions.

To eliminate this problem we added ornithine transcarbamylase to the reaction mixture to enzymatically remove the carbamoyl phosphate that was produced. Thus, the only reversal possible is from the substrates bound at the active site of the enzyme. With the inclusion of NH_4^+ and ornithine transcarbamylase; there was no detectable positional isotope exchange as predicted from the ordered reaction mechanism as previously published by us (Raushel et al., 1978).

The kinetic scheme for the ATPase reaction (Raushel & Villafranca, 1979) can now be revised by using the new rate for the positional isotope exchange reaction under our experimental conditions. The ATPase reaction is presented in the simple scheme

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \xrightarrow{k_5} E + P$$

In this scheme EA represents the enzyme–ATP–bicarbonate complex and EP represents the enzyme–ADP–carboxy phosphate complex. The following relationships hold:

$$k_{cat} = \frac{k_3 k_5}{k_3 + k_4 + k_5} = 0.20\ s^{-1}$$

$$\lambda = k_3 + k_4 + k_5 = 4.5\ s^{-1}$$

$$\frac{k_x}{k_{cat}} = \frac{k_2 k_4}{k_5(k_2 + k_3)} = 0.46$$

In this set of equations k_{cat} is the steady-state rate for the ATPase reaction and λ is the transient rate constant for the "burst" of acid-labile phosphate from rapid-quench experiments (Raushel & Villafranca, 1979). k_x/k_{cat} is the ratio of the positional isotope exchange reaction rate to the chemical rate. The values computed for k_3, k_4, and k_5 are 4.2 s^{-1}, 0.10 s^{-1}, and 0.21 s^{-1}. The value for k_2 has been shown to be $\gg$3.1 s^{-1} (Raushel & Villafranca, 1979). Thus, k_5 is limiting for the ATPase reaction and k_3 (the rate constant for the formation of carboxy phosphate) is probably rate limiting for the overall reaction in the presence of glutamine since the k_3 value is very close to k_{cat} for the overall reaction (3.1 s^{-1}) (Raushel & Villafranca, 1979).

In summary, we have shown using ^{31}P NMR that the positional isotope exchange rate in the partial reverse reaction of carbamoyl-phosphate synthetase is 4 times the steady-state rate for the synthesis of ATP. This indicates that the rate-limiting step is after the bond breaking step in this reaction. This step is most likely the release of carbamate or a rate-limiting conformational change permitting such release. In the ATPase reaction the positional isotope exchange rate is 0.46 times as fast as the chemical reaction rate. This information, in addition to previously published results, shows that the release of carboxy phosphate is limiting the ATPase reaction and the synthesis of carboxy phosphate probably limits

the overall synthesis of carbamoyl phosphate.

The use of ^{31}P NMR appears to be the method of choice for the measurement of positional isotope exchange reactions in phosphate esters. The amount of material used in these studies is about the same as that used with the mass spectral method, and there is no need for extensive degradation of the products. The major advantage of the NMR method is that the reaction can be followed continuously if adequate sensitivity and resolution are obtained.

Acknowledgments

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Structure of the Cytochrome *c* Oxidase Complex: Labeling by Hydrophilic and Hydrophobic Protein Modifying Reagents†

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ABSTRACT: Beef heart cytochrome *c* oxidase has been reacted with [^{35}S]diazobenzenesulfonate ([^{35}S]DABS), [^{35}S]-*N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate ([^{35}S]NAP-taurine), and two different radioactive arylazidophospholipids. The labeling of the seven different subunits of the enzyme with these protein modifying reagents has been examined. DABS, a water-soluble, lipid-insoluble reagent, reacted with subunits II, III, IV, V, and VII but labeled I or VI only poorly. The arylazidophospholipids, probes for the bilayer-intercalated portion of cytochrome *c* oxidase, labeled I, III, and VII heavily and II and IV lightly but did not react with V or VI. NAP-taurine labeled all of the subunits of cytochrome *c* oxidase. Evidence is presented that this latter reagent reacts with the enzyme from outside the bilayer, and the pattern of labeling with the different hydrophilic and hydrophobic labeling reagents is used to derive a model for the arrangement of subunits in cytochrome *c* oxidase.

Cytochrome *c* oxidase is the terminal member of the electron transport chain, an integral part of coupling site III, and an intrinsic component of the mitochondrial inner membrane. The protein complex contains two heme moieties (*a* and a_3) and two copper atoms as electron acceptors along with seven (or possibly more) polypeptides in a complex of molecular weight around 140 000 [for reviews, see Erecinska & Wilson (1978) and Capaldi (1979)]. Recently, considerable progress has been made in determining the structure of this complex. The gross shape and approximate size of cytochrome *c* oxidase from beef heart mitochondria has been obtained by electron microscopy and image reconstruction studies (Henderson et al., 1977; Fuller et al., 1979). The protein is seen as Y shaped and made up of three domains, two of which (the M_1 and M_2 domains) span the lipid bilayer; the third (or C domain) is outside the bilayer (Fuller et al., 1979). Cytochrome *c* binding (S. D. Fuller and R. A. Capaldi, unpublished results) and antibody binding experiments (Frey et al., 1978) indicate that the C domain is located on the cytoplasmic side of the mitochondrial inner membrane (hence the nomenclature); the two M domains extend a small way into the matrix space. The arrangement of the subunits in cytochrome *c* oxidase has been examined by reacting the enzyme with [^{35}S]DABS (Eytan & Schatz, 1975; Eytan et al., 1975; Eytan & Broza, 1978; Ludwig et al., 1979), by lactoperoxidase-catalyzed iodination of the complex (Eytan & Schatz, 1975), by antibody binding experiments (Chan & Tracy, 1978), and by using iodoaryl azides (Cerletti & Schatz, 1979) and arylazidophospholipids (Bisson et al., 1979a,b). The orientation of the enzyme in the mitochondrial inner membrane has also been explored by labeling with [^{35}S]DABS (Eytan et al., 1975; Ludwig et al., 1979) and by antibody binding (Chan & Tracy, 1978).

The concensus from these studies is that subunits II and III are on the cytoplasmic side of the inner membrane and thus a part of the C domain of the cytochrome *c* oxidase complex. Subunit IV is generally accepted to be on the matrix side of the membrane, while subunit I is considered to be predomi-

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