Mechanism of Mitochondrial Carbamoyl-Phosphate Synthetase. Synthesis and Properties of Active CO_2, Precursor of Carbamoyl Phosphate†

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ABSTRACT: This paper demonstrates the formation of "active CO_2" (CO_2-P), a precursor of carbamoyl phosphate (CP), with frog liver carbamoyl-phosphate synthetase. Absence of ammonia is essential for the demonstration by pulse incubation with $H^{14}CO_3^-$ of CO_2-P. Adenosine triphosphate (ATP) and acetylglutamate are required for the synthesis of CO_2-P, which is highly unstable in aqueous solutions ($t_{1/2}$ = 0.75 s at 24 °C at neutral pH). In the absence of ammonia, CO_2-P attains rapidly a steady-state level, which depends on the concentration of ATP and HCO_3^-. The "apparent K_M's" are approximately equal to those found for the adenosine triphosphatase (ATPase) activity of the enzyme. The maximum level of CO_2-P is limited by the amount of enzyme, and approximates 4 mol of intermediate/mol of enzyme. The unprotonated form of ammonia seems to be the species reacting with CO_2-P to produce CP. The reaction of CO_2-P and NH_3 is very fast (rate constant $k_n = 8 \times 10^4$ M^{-1} s^{-1}) and does not consume free ATP. Therefore, the 2 mol of ATP necessary for CP synthesis binds or reacts with the enzyme and/or CO_2 prior to reaction with NH_3. The reaction of CO_2-P with NH_3 also takes place in acetone under conditions at which the enzyme is not active, suggesting little or no assistance from enzyme catalysis or that a part of the catalytic site is "frozen" by the solvent in the active conformation. In the light of these and other findings, a new scheme is proposed for the mechanism of frog liver carbamoyl-phosphate synthetase and some considerations are made on the chemical nature of the intermediate and on the possible evolutionary significance of the reaction of CO_2-P with NH_3 in acetone.

After nearly 2 decades of essentially classical enzymology, the main enzymes of the urea cycle can be obtained with high purity and/or homogeneity and the mechanisms of most of these enzymes are largely understood with the remarkable exception, and in spite of extensive investigations, of carbamoyl-phosphate synthetase, an enzyme which represents from 20–60% of the total protein of liver mitoplasts of ureotelic animals (Raijman, 1976).

The AG[1] and bicarbonate-dependent hydrolysis of ATP catalyzed by frog liver carbamoyl-phosphate synthase in the absence of ammonia was interpreted as evidence for the formation of "active CO_2" as a first step (Metzenberg et al., 1958). Subsequently, the intermediate was formulated as enzyme-bound carbonyl phosphate, since bicarbonate contributes one oxygen atom to phosphate (Jones, 1965), although there has been little experimental clarification of the overall mechanism which is generally agreed to occur as per reactions 1–3.

$$E + HCO_3^- + ATP \xrightarrow{AG,\ Mg^{2+},\ K^+} E \cdot CO_3PO_3^{3-} + ADP \quad (1)$$

$$E \cdot CO_3PO_3^{3-} + NH_4^+ \xrightarrow{AG,\ Mg^{2+}} E \cdot CO_2NH_2^- + P_i \quad (2)$$

$$E \cdot CO_2NH_2^- + ATP \overset{AG,\ Mg^{2+}}{\rightleftharpoons} NH_2CO_2PO_3^{2-} + ADP + E \quad (3)$$

Studies with carbamoyl-phosphate synthetase from *Escherichia coli*, which also catalyzes a bicarbonate-dependent ATPase but does not require acetylglutamate (Anderson and Meister, 1966), provided evidence by pulse labeling with $H^{14}CO_3^-$ (Anderson and Meister, 1965) for such an enzyme-bound intermediate. However, McKinley et al. (1967) mentioned that similar experiments with the frog enzyme were negative. While it is generally agreed that an "active CO_2" is also a CP precursor with animal carbamoyl-phosphate synthetase, even well-designed experiments have failed to clarify its identity. Indeed, Guthöhrlein and Knappe (1969) indicated that, since the elusive active CO_2 may have a half-life of ~0.6–2 s, pulse-label experiments may not be successful with mitochondrial carbamoyl-phosphate synthetase. The existence of an enzyme-bound carbonyl phosphate has been suggested also for a number of carboxylation reactions requiring biotin (Moss and Lane, 1971; Kaziro et al., 1962).

Kinetic studies indicate no irreversible step between the addition of HCO_3^- and NH_4^+ to carbamoyl-phosphate synthetase (Guthöhrlein and Knappe, 1969) and that the first product released is CP (Elliott and Tipton, 1974a,b). As pointed out by Marshall (1976), these results are not necessarily in conflict with reactions 1–3. The rate of the ATPase is slow compared with that of the overall reaction, so that ADP may not dissociate before the addition of ammonia. Carbamoyl-phosphate synthetase also catalyzes the synthesis of ATP from CP and ADP. One mole of ATP is formed per mole of CP used (Metzenberg et al., 1958); thus, it was proposed that this reaction is the reverse of equation 3 (Metzenberg et al. 1958; Jones, 1965). However, as formulated, it does not fit into Elliott

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[1] Abbreviations used are: AG or acetylglutamate, *N*-acetyl-L-glutamate; CP, carbamoyl phosphate; E, carbamoyl-phosphate synthetase (from frog liver unless specified); P_i, orthophosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NADH, reduced nicotinamide adenine dinucleotide.

and Tipton's scheme for ordered product release. Moreover, as indicated by Chabas et al. (1972) and by Jones (1976), such a partial reaction does not seem to be truly a reversal of the forward reaction. Also, as shown by Tate et al. (1972) glutamine synthetase catalyzes such a reaction, although it does not catalyze the synthesis of CP, and Polakis et al. (1972) have shown a similar reaction with biotin carboxylase. Therefore, reversibility of reaction 3 in the context of being a partial reaction for carbamoyl-phosphate synthetase is probably trivial.

This paper presents experimental evidence for ATP-dependent formation of an activated CO_2 intermediate by carbamoyl-phosphate synthetase from frog liver, some properties of this intermediate, and a new scheme for the mechanism of CP synthesis.

Materials and Methods

Chemicals. $NaH^{14}CO_3$, [γ-^{32}P]ATP, tetra(triethylammonium) salt, and Aquasol (scintillation solution) were obtained from New England Nuclear. All other chemicals were of the purest grade commercially available.

Enzymes. Pyruvate kinase [EC 2.7.1.40] (rabbit muscle, type III, lyophilized, ammonium free) and lactic acid dehydrogenase [EC 1.1.1.27] (rabbit muscle, type XI, lyophilized, ammonium free) were purchased from Sigma.

Frog (Bullfrog, *Rana Catesbeiana*) liver carbamoyl-phosphate synthetase was prepared by the method of Marshall et al. (1958). After the cellulose phosphate chromatography step, fractions containing higher specific activity were pooled. Usually, the enzyme was concentrated by XM-50 ultrafiltration (Amicon); for small volumes, dry Sephadex G-25 coarse was used as described by Kibukamusoke and Wilks (1965). No AG-independent ATPase, ornithine transcarbamylase, or adenylate kinase were detected. The preparation was 85–90% pure on the basis of the specific activity for the allegedly pure carbamoyl-phosphate synthetase from frog liver (Strahler, 1974). Purity was also monitored by disc electrophoresis (Guthöhrlein and Knappe, 1968) and by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Clarke, 1976). By the first method, 90% protein (as integrated A_{590nm} of amido black in the densitometric trace obtained with a Gilford gel scanner) migrated as a single band with an R_f of 0.47 relative to the tracking dye. In addition, two minor bands (1 and 9% with R_f of 0.37 and 0.59, respectively) were seen. Only the major band showed carbomyl-phosphate synthetase activity when assayed as described below. By sodium dodecyl sulfate gel electrophoresis, 88% of the protein (as integrated A_{520nm} of Coomasie blue in the densitometric trace) migrated as a single band. Five other bands with mobilities relative to the main band equal to 1.07, 1.14, 1.22, 1.33, and 2.15 accounted for 3.5, 3, 2, 2, and 1.5%, respectively, of the protein. The size and number of the small bands increased on prolonged storage at 4 °C. The mobility of the main band was 0.51 relative to that of bovine serum albumin (Sigma) monomer.

Enzyme Assays. Carbamoyl-phosphate synthetase and ATPase activities were measured at 37 °C, as described by Chabas et al. (1972). Under these conditions, assuming a mol wt for carbamoyl-phosphate synthetase of 320 000 D and the activity for pure enzyme (Strahler, 1974), an AG-dependent change in A_{340} of 0.9/min represents 0.1 nmol of enzyme. Ornithine transcarbamylase and adenylate kinase were determined by the method of Caravaca and Grisolia (1960) and of Schnaitman and Greenawalt (1968), respectively. Carbamoyl-phosphate synthetase was assayed on polyacrylamide gels by incubating the gels for 30 min at 24 °C in potassium glycylglycine, 0.05 M, ATP, 5 mM, AG, 10 mM, $KHCO_3$, 50 mM, $MgSO_4$, 15 mM, and mercaptoethanol, 5 mM, pH 7.4 (controls without AG were carried out), and then rinsed in water and developed for P_i (Gabriel, 1971).

Separation of $^{32}P_i$ from [γ-^{32}P]ATP. The following modification of the Berenblum and Chain method (1938) was used: to ~0.7 ml of solution, 1 ml of 1 N H_2SO_4, 0.2 ml of 5% ammonium molybdate, and 2 ml of 2-butanol were added in rapid succession, using automatic syringes, and mixed by bubbling air for 15 s. After 60 s, the 2-butanol layer was rapidly taken and washed with 1 ml of 0.5 N H_2SO_4. The samples were mixed with 10 ml of Aquasol and counted.

Synthesis and Quantitation of CO_2-P. Unless otherwise indicated, all incubations were at 24 °C and all solutions were adjusted to pH 7.4 with KOH as necessary. Bicarbonate solutions were adjusted to pH 7.4 by bubbling with CO_2.

Pulse-label experiments were performed essentially as described by Anderson and Meister (1965). However, since it is crucial to remove trace amounts of ammonia, any contaminating ammonia (usually less than 25 nmol/tube) was converted into nonradioactive CP by short incubation of the enzyme, buffer, AG, Mg^{2+}, ATP, and HCO_3^-, before addition of $H^{14}CO_3^-$. Other experimental details, reagent concentrations, etc. are given below. For comparative purposes with the work of Anderson and Meister (1965), the pulse-chase was used for some experiments. However, the procedure was further modified to include a dilution in volume, and, therefore, in concentration, by taking the entire mixture into a Pasteur pipet after addition of $H^{14}CO_3^-$ and at 60 s (measured from the moment of $H^{14}CO_3^-$ addition) squirting it at once into 10 ml of a rapidly stirring solution containing buffer, ammonia, and/or other components (see below). Equal values were obtained with or without dilution. However, the pulse-dilution technique is preferable in many cases, for it slows rapidly the reaction and facilitates experimental changes in pH, μ, substrate, or activator concentrations, etc. It should be noted that the mixing was found experimentally to be instantaneous. The reaction was stopped by addition of 0.2 ml of 3 N KOH to the mixture (or when experiments included the dilution in volume step, to a 0.2-ml aliquot); 0.2 ml of 0.001 M CP was then mixed in.

CP was converted to urea by a modification of the procedure of Allen and Jones (1964) as follows: the mixture was incubated for 10 min at 37 °C, and after addition of 0.4 ml of 4 M NH_4Cl, pH 8.5, it was transferred to a boiling water bath for 10 min, cooled, and acidified with concentrated HCl and N_2 was bubbled through for 2 min. This treatment removed all bicarbonate. The acid-resistant radioactivity remaining in solution could be removed with urease. There was complete conversion of CP to urea.

Other methods. Protein was quantitated by the method of Lowry et al. (1951), or by measuring the absorbance at 280 nm using an $E_{280nm}^{1\%} = 9.6$ for E in 0.02 M Tris-Cl$^-$, pH 7.5 (Fahien and Cohen, 1964). Urea was measured by the colorimetric method of Hunninghake and Grisolia (1966).

$NaH^{14}CO_3$ was measured in 1 ml of hydroxide of hyamine 10-X. The solubilizer containing the bicarbonate was dissolved in 10 ml of Aquasol and was counted in a tightly closed vial (usually after a few hours in the dark) against a blank prepared in the same way but containing no bicarbonate.

Results

Formation of an Active CO_2 Intermediate with Frog Liver Carbamoyl-Phosphate Synthetase. Evidence for the formation of an active CO_2 intermediate as the putative precursor of CP

TABLE I: Need for AG, HCO_3^-, and ATP for CO_2-P Intermediate Formation with Carbamoyl-Phosphate Synthetase.[a]

Acetyl-glutamate Added	Components added during stage A	B	C	Radioactivity (cpm)
+	HCO_3^-, ATP	$H^{14}CO_3^-$	HCO_3^-, NH_3	76556
+	HCO_3^-, ATP	$H^{14}CO_3^-$	HCO_3^-, NH_3[b]	2018
−	HCO_3^-, ATP	$H^{14}CO_3^-$	HCO_3^-, NH_3	2697
+	HCO_3^-	$H^{14}CO_3^-$	HCO_3^-, NH_3, ATP	3082
+	ATP		HCO_3^-, NH_3, $H^{14}CO_3^-$	3300

[a] 2.3 nmol of enzyme, 11.4 μmol of potassium glycylglycine, 10 μmol of $MgCl_2$, and, when indicated, 2.5 μmol of AG in 100 μl were incubated for 10 min. Then, 40 μl of 0.1 M ATP and/or 5 μl of 0.1 M $KHCO_3$ were added and the mixtures were incubated for 2.5 min (stage A). 5 μl of 0.01 M $NaH^{14}CO_3$ containing 6×10^6 cpm was then added (when indicated), and the incubation was continued for 30 s (stage B). At this point, the indicated components for stage C ($KHCO_3$, 1 M, 100 μl; $(NH_4)_2SO_4$, 0.7 M, 10 μl; ATP, 0.1 M, 40 μl; $NaH^{14}CO_3$, 0.01 M, 5 μl, 6×10^6 cpm) were added at once as a mixture. The reaction was stopped after exactly 10 s with 0.2 ml of 3 N KOH, and the entire mixture was treated as described under Methods to convert CP to urea. [b] KOH was added before mixing in the components of stage C.

TABLE II: Conversion of CO_2-P to CP in the Presence of ATP, ADP, AG, HCO_3^-, Mg^{2+} and in Acetone.[a]

Solvent	Final Concentration (mM) of ATP	ADP	AG	HCO_3^-	Mg^{2+}	CP Found (nmol)
Water	5.1		10.1	49.3	15.4	5.8
	0.2		10.1	49.3	15.4	5.7
	0.2	9.9	10.1	49.3	15.4	5.9
	0.2		0.25	49.3	15.4	5.8
	0.2		0.25	0.05	15.4	5.9
	0.2		0.25	0.05	0.6	5.9
Acetone	0.2		0.25	0.05	0.6	5.7

[a] Two nanomoles of enzyme, 11.4 μmol of potassium glycylclycine, 6 μmol of $MgCl_2$, and 2.5 μmol of AG in 120 μl were incubated for 10 min. Then, 20 μl of 0.1 M ATP and 5 μl of 0.1 M $KHCO_3$ were added. Five minutes later, 5 μl of 0.01 M $NaH^{14}CO_3$ containing 6×10^6 cpm were mixed in, and after 60 s it was rapidly mixed into 10 ml of a 0.05 M solution of potassium glycylglycine buffer or acetone (which were kept stirring mechanically) containing 350 μmol of $(NH_4)_2SO_4$ and the indicated components to give (after mixing in the enzyme solution) the final concentrations indicated in the table. Ten seconds after dilution, 0.2-ml aliquots were withdrawn from the mixtures, combined with KOH and CP, and treated as described under Methods to convert CP to urea. Controls were carried in an identical way, except that the $H^{14}CO_3^-$ was added to the diluting solution. Acetone did not affect the degree of conversion of CP to urea.

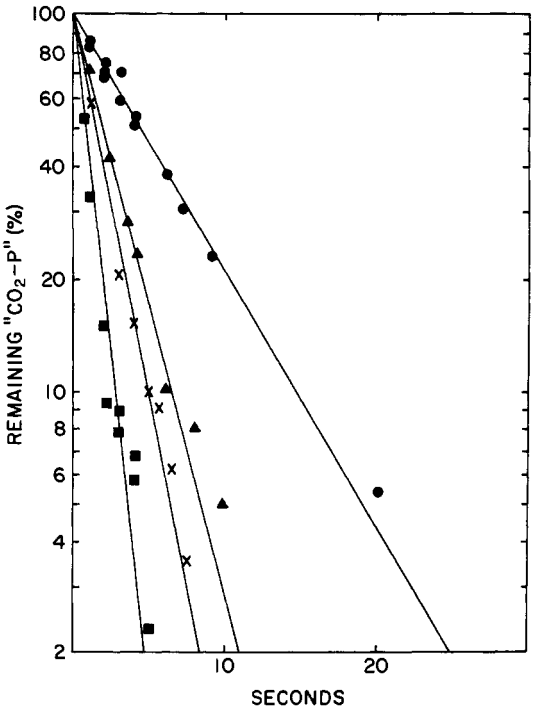


FIGURE 1: Kinetics of disappearance of CO_2-P at different temperatures. Two nanomoles of enzyme, 11.4 μmol of potassium glycylglycine, 6 μmol of $MgCl_2$, and 2.5 μmol of AG in 120 μl were incubated for 10 min. Then, 20 μl of 0.1 M ATP and 5 μl of 0.1 M $KHCO_3$ were added. Five minutes later, 5 μl of 0.01 M $NaH^{14}CO_3$, containing 6×10^6 cpm, was mixed in, and after 60 s the entire mixture was rapidly stirred into 10 ml of 0.05 M potassium glycylglycine–15 mM $MgCl_2$–50 mM $KHCO_3$, kept at 0 (●), 10 (▲), 14 (+), or 24 °C (■). At the indicated times after dilution, 0.5 ml of 0.7 M $(NH_4)_2SO_4$ was mixed in rapidly, and 10 s later a 0.2-ml aliquot was withdrawn from the mixture, combined immediately with koh and CP, and treated as described under Methods to convert CP to urea. All values have been corrected for the very small production of CP of low specific activity during the 10-s "trapping" with ammonia by substracting the values from samples left to decay for 60–90 s before the addition of ammonia.

with frog liver carbamoyl-phosphate synthetase was obtained by pulse-label experiments. These results, illustrated in Table I, clearly demonstrate ATP- and AG-dependent formation of an intermediate which subsequently reacts with ammonia to generate CP. Although the actual structure of the intermediate is as yet unknown, for convenience from here on it will be referred to as CO_2-P. The possibility that CP of high specific activity was produced during the "pulse" portion of the incubation was eliminated by the low incorporation of radioactivity when KOH was added before ammonia.

As illustrated in Table II, the conversion of CO_2-P to CP was not affected by addition of extra amounts of ATP, HCO_3^-, Mg^{2+}, and AG. The addition of ADP, an inhibitor of CP synthesis (Kerson and Appel, 1968) mainly by competition with ATP, at high ADP/ATP ratios was without effect. Ammonia was the only substrate which affected the conversion of CO_2-P to CP (see below). Interestingly, when the dilution was made in acetone instead of buffer, similar amounts of CP were obtained. These findings suggest that ammonia is the only compound which reacts with CO_2-P to produce CP and that an active carbamoyl-phosphate synthetase may not be necessary for the reaction to proceed.

Stability of CO_2-P. Figure 1 shows that CO_2-P disappears following pseudo-first-order kinetics. The apparent first-order rate constant (k_d) is too large to be accurately measured at 37 °C (standard temperature at which most kinetic and mechanism studies with carbamoyl-phosphate synthetase have been

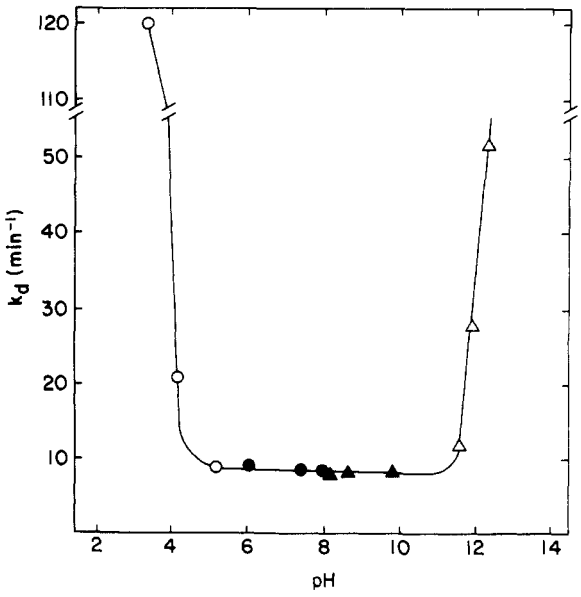


FIGURE 2: Observed first-order rate constants for the disappearance of CO_2-P at 0 °C as a function of pH. Three nanomoles of enzyme, 2.5 μmol of potassium glycylclygine, 6 μmol of $MgCl_2$, and 2.5 μmol of AG in 125 μl were incubated for 10 min. Then, 10 μl of 0.1 M ATP and 5 μl of 0.1 M $KHCO_3$ were added. Five minutes later, 5 μl of 0.01 M $NaH^{14}CO_3$ containing 6×10^6 cpm was added, and after 60 s the entire solution was rapidly mixed into 10 ml of ice-cold solutions of 0.005 M sodium citrate buffer (○), potassium phosphate buffer (●), Tris-Cl^- buffer (▲), or 0.002, 0.005, or 0.01 M NaOH (△, ascending pH's). The indicated pH's were measured at 0 °C after adding the enzyme solution. In all cases, all solutions contained enough KCl to give 0.2 μ in the final mixture. At appropriate times after dilution, 1 ml of 0.35 M $(NH_4)_2SO_4$–1 M potassium phosphate buffer, pH 7.4, was added, and 10 s later a 0.2-ml aliquot was combined with KOH and CP and treated as described under Methods to convert CP to urea.

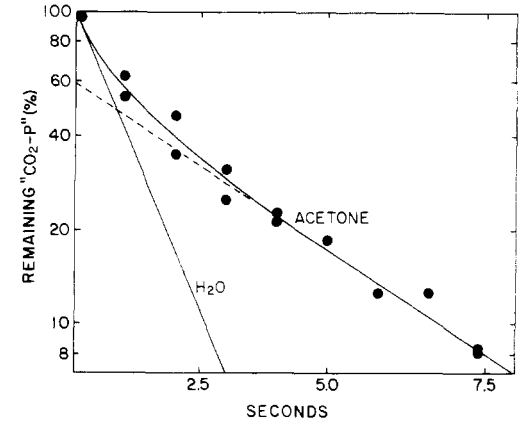


FIGURE 3: The effect of acetone on the degradation of CO_2-P. Two nanomoles of enzyme, 11.4 μmol of glycylglycine, 6 μmol of $MgCl_2$, and 2.5 μmol of AG in 120 μl were incubated for 10 min. Then 20 μl of 0.1 M ATP and 5 μl of 0.1 M $KHCO_3$ were added. Five minutes later, 5 μl of 0.01 M $NaH^{14}CO_3$ containing 6×10^6 cpm was mixed in, and after 60 s the entire mixture was rapidly stirred into 10 ml of acetone kept at 24 °C. At the indicated times after dilution, 0.5 ml of 0.7 M $(NH_4)_2SO_4$ was mixed in rapidly, and 10 s later a 0.2-ml aliquot was withdrawn from the mixture, combined immediately with KOH and CP, and treated as described under Methods to convert CP to urea. For comparative purposes, the effect of time on the degradation of CO_2-P in H_2O is shown. The curved part of the line is interpreted as due to the slow mixing of acetone with the water trapped in the protein precipitate.

performed) without the use of rapid mixing instrumentation. However, k_d could be measured with reasonable accuracy at 24 °C and below. The k_d values at 0, 10, 14, and 24 °C were 9.3, 20.9, 27.8, and 55.6 min^{-1}. The Arrhenius plot of ln k_d at these temperatures proved to be linear (calculated activation energy, E_a = 12.1 kcal/mol) giving by extrapolation values for k_d at 25 and 37 °C of 60 (1 s^{-1}) and 138 min^{-1} (2.3 s^{-1}), respectively. These values are within one order of magnitude of the calculated nonenzymatic rate of decomposition ($k_d \geq 10$ s^{-1}) in dilute alkali solutions of the hypothetical carbonyl phosphate intermediate (Sauers et al., 1975). As shown in Figure 2, k_d for "CO_2-P" increases rapidly above pH 11, making closer the agreement between these values. Figure 2 also illustrates that no marked changes in the magnitude of k_d occur between pH's 5 and 11, opposite to what would be expected if different ionic species of the same molecule decomposed at different rates (as is the case with acetyl phosphate (Koshland, 1952)). Below pH 5, k_d increases again, suggesting acid catalysis.

The general form for the rate curve is similar to that described by Allen and Jones (1964) for CP hydrolysis, except for the absence of measurable rate changes in the flat portion and the shift of the entire curve of about 2 pH units towards the basic side.

From 0.6 mM to 0.1 M Mg^{2+} does not affect the rate constant, unlike the case of acetyl phosphate (Oestreich and Jones, 1966a) and CP (Oestreich and Jones, 1966b), suggesting different mechanisms of decomposition.

When CO_2-P is left to decay in acetone ($[H_2O] \simeq 0.8$ M) at 24 °C (Figure 3), the half-life increases to 2.8 s. The remaining CO_2-P can be converted to CP any time after addition of the enzyme mixture to the acetone. Under these conditions, the enzyme is inactive; i.e., it does not catalyze CP or CO_2-P synthesis from ammonia, HCO_3^-, and ATP, indicating that the interaction of CO_2-P and ammonia to produce CP proceeds with little or no catalysis by the enzyme.

Dependency of CO_2-P Synthesis on the Concentrations of ATP and HCO_3^-. The amount of CO_2-P produced by a given quantity of enzyme depends on the concentrations of ATP and HCO_3^-. When the reciprocal of CO_2-P accumulated vs. the reciprocal of the concentrations of ATP and bicarbonate used was graphed, linear plots were obtained. This would be expected for an enzyme-bound intermediate which determines the rate of the reaction, according to eq 4.

$$v = k'[CO_2\text{-P}] \quad (4)$$

Apparent K_M^{ATP} and $K_M^{HCO_3^-}$ of 0.18 and 2.4 mM, respectively, were calculated from the plots. The first value agrees with the K_M^{ATP} (0.18 mM) reported by Fahien and Cohen (1964) from the rate of ADP liberation by carbamoyl-phosphate synthetase in the absence of ammonia. No accurate $K_M^{HCO_3^-}$ has been published for frog carbamoyl-phosphate synthetase; we have obtained values of 1.5–4.5 mM (at saturating ammonia) by the spectrophotometric procedure described above. Thus, the apparent $K_M^{HCO_3^-}$ given for CO_2-P synthesis is within this range.

Dependency of CO_2-P Synthesis on the Amount of Enzyme. The amount of radioactive CO_2-P increases rapidly after the addition of $H^{14}CO_3^-$, reaching a plateau of 5 s under the conditions described in the legend of Figure 4. The amount of $^{14}CO_2$-P synthesized at 2 s approaches the expected value according to eq 5.

$$(CO_2\text{-P})_s = (CO_2\text{-P})_\infty(1 - e^{-kt}) \quad (5)$$

(where $(CO_2\text{-P})_s$ = amount of intermediate formed at s seconds; $(CO_2\text{-P})_\infty$ = amount of intermediate at the plateau) for a value of k = 0.93 s^{-1} = 55.8 min^{-1} $\simeq k_d$ for CO_2-P at 24 °C.

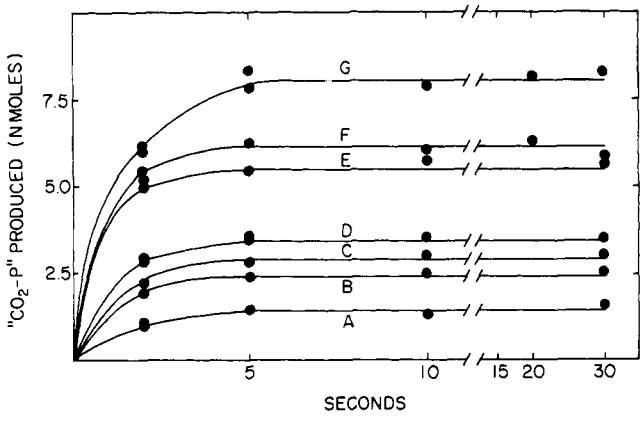


FIGURE 4: Synthesis of CO_2-P as a function of time and amount of enzyme. For curves A–G, respectively, 0.4, 0.66, 0.84, 1.15, 1.66, 1.93, and 2.5 nmol of enzyme, 12.5 μmol of potassium glycylglycine, 2.5 μmol of AG, and 6 μmol of $MgCl_2$ in 120 μl were incubated for 10 min. Then 10 μl of 0.1 M ATP and 20 μl 0.1 M $KHCO_3$ were added. Five minutes later, 5 μl of 0.01 M $NaH^{14}CO_3$ containing 6×10^6 cpm was mixed in, and, after the indicated periods of time, 100 μl of 1 M $KHCO_3$–0.07 M $(NH_4)_2SO_4$ was added at once. The reaction was stopped after 10 s with 0.2 ml of KOH, and the entire mixture was treated as described under Methods to assay CP by conversion to urea. Controls were performed by running samples in an identical way, except that $H^{14}CO_3^-$ was added to the "trapping" solution.

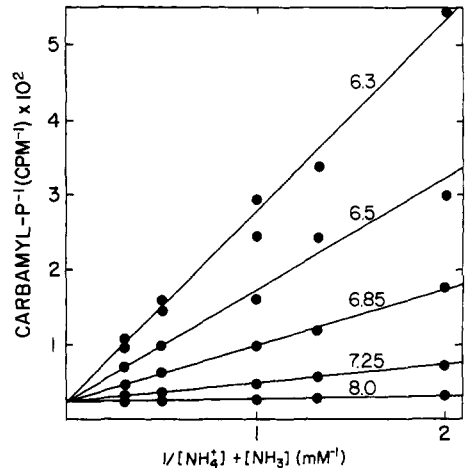


FIGURE 5: Synthesis of CP from CO_2-P as a function of pH and ammonia concentration. 0.35 nmols of enzyme, 2.5 μmol of potassium glycylglycine, 6 μmol of $MgCl_2$, and 2.5 μmol of AG in 120 μl were incubated for 10 min. Then, 10 μl of 0.1 M ATP and 5 μl of 0.1 M $KHCO_3$ were added and 5 min later 5 μl of 2 μM $NaH^{14}CO_3$ (1×10^6 cpm) was mixed in. After 60-s samples of such mixtures were rapidly diluted in 10 ml of potassium phosphate buffer, 0.005 M, at the indicated pH's, containing the indicated amounts of $(NH_4)_2SO_4$ and KCl to give a concentration of 0.2 μ (pH and μ refer to the final mixture after the addition of the enzyme solution). Ten seconds after dilution, 0.2 ml of the mixture was combined with 0.2 ml of 3 N KOH and 0.2 ml of 0.001 M CP and converted to urea, as described under Methods. All values have been corrected for the small production of CP during the 10-s "trapping" with ammonia (values obtained with samples where radioactive bicarbonate was added to the "trapping" solution). The pH's of the buffers used are indicated in the figure.

TABLE III: ATP Utilization During CP Formation from CO_2-P and Ammonia.[a]

ATP Present During Incubation	Time (s)	Radioactivity (cpm)[b] Due to P_i Released	
		Expected	Found
−	10		2711
−	20		2417
−	30		2854
+	10	6200[c]	2433
+	20	6200	2237
+	30	6200	2183

[a] Three nanomoles of enzyme, 12.5 μmol of potassium glycylglycine, 6 μmol of $MgCl_2$, and 2.5 μmol of AG in 125 μl were incubated for 10 min. Then 10 μl of 0.1 M ATP (when indicated) and 10 μl of 0.1 M $KHCO_3$ were added, and 6 min later the mixture was rapidly diluted into 10 ml of a well-stirred solution (kept at 0 °C) of 0.05 M potassium glycylglycine, 0.035 M $(NH_4)_2SO_4$ containing 3 nmol of [γ-^{32}P]ATP (17.7×10^6 cpm) and for controls (ATP absent during the incubation) 1 μmol of ATP. At the indicated times after dilution, 0.2-ml aliquots were withdrawn, combined with KOH and CP, heated for 10 min at 37 °C, and then kept at 22 °C for 10 more min (each sample was extracted in succession to minimize nonenzymatic liberation of P_i during manipulation). After neutralization with 5 N HCl, $^{32}P_i$ was extracted and measured as described under Methods. [b] In 0.2-ml aliquot. [c] Parallel experiments with $H^{14}CO_3^-$ showed production of 10 nmol of CO_2-P. This would correspond to 3550 cpm of $^{32}P_i$ over the controls if 1 mol of [^{32}P]ATP was used to convert 1 mol of CO_2-P to CP. Therefore, the expected radioactivity for the samples incubated with ATP, if this reagent is used during conversion of CO_2-P to CP, is ~6200 cpm.

Figure 4 also shows that the maximum accumulation of CO_2-P is proportional to the concentration of enzyme. By plotting the amount of CO_2-P at the plateau vs. the amount of enzyme, a straight line of zero intercept and 3.35 (mol of CO_2-P/mol of enzyme) slope is obtained for the experimental conditions given in the legend of Figure 4. The calculated ratio at saturating substrate concentrations is 4.1.

Conversion of CO_2-P to CP. Results presented in Table III show that when [γ-^{32}P]ATP together with ammonia as a single mixture is added to CO_2-P to produce CP there is no $^{32}P_i$ released. Therefore, the two ATP molecules needed for CP synthesis bind to the enzyme (or react with the enzyme and/or CO_2) in the presence of CO_2 prior to reaction with NH_3 and do not equilibrate with added [^{32}P]ATP. Perhaps both ATP molecules are involved in the synthesis of CO_2-P which then would have to react only with ammonia to produce CP.

The conversion of CO_2-P to CP on reaction with ammonia is very fast. At the NH_3 concentration used in the experiments reported in Table I, the production of CP was practically instantaneous. Moreover, the reaction proceeds to a large extent (50–75% of completion), even at very basic pH (1.35 N KOH), despite the low stability of CO_2-P at high pH's and the rapid denaturation of the enzyme under these conditions.

To ascertain which species, NH_3 or NH_4^+, is used in the conversion of CO_2-P to CP, we performed the experiments depicted in Figure 5. The rationale for them was the assumption that the ratio of the amounts of CO_2-P converted to CP or decomposed in a solution containing ammonia will be equal (when all CO_2-P has disappeared) to the ratio of the apparent pseudo-first-order rate constants of the respective reactions (eq 6a).

$$\frac{k_d}{k_n'} = \frac{k_d}{k_n[S]} = \frac{CO_2\text{-}P - CP}{CP} \qquad (6a)$$

where CP = carbamoyl phosphate produced; CO_2-P = initial amount of intermediate; k_n' = pseudo-first-order rate constant for CP synthesis from CO_2-P at a fixed [S]; k_n = second-order-rate constant for CP synthesis from CO_2-P and ammonia; S = NH_3 or NH_4^+. Reordering and putting in the reciprocal form, eq 6a yields eq 6b.

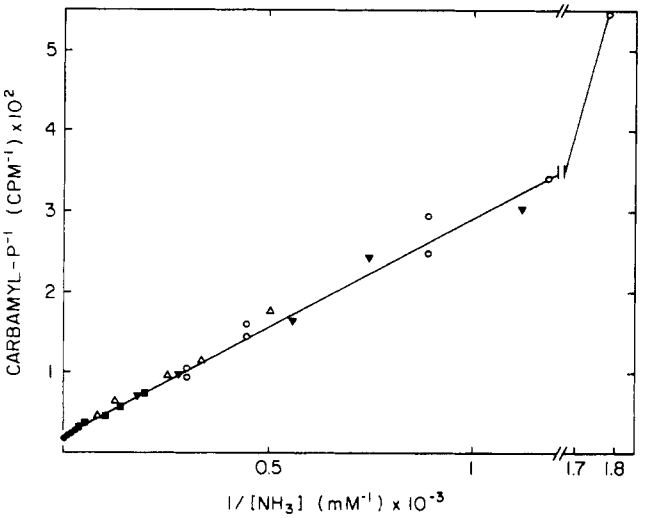


FIGURE 6: Synthesis of CP from CO_2-P as a function of NH_3 concentration. Same points of Figure 5 plotted against the reciprocal of the concentration of NH_3. (●) pH 8.0; (■) pH 7.25; (△) pH 6.85; (▼) pH 6.5; (○) pH 6.3.

$$\frac{1}{CP} = \frac{1}{CO_2\text{-}P} + \frac{1}{CO_2\text{-}P}\frac{k_d}{k_n}\frac{1}{[S]} \qquad (6b)$$

The ionic state of the substrate can be tested by changing the pH within a range far from the pK_a of NH_4^+ towards the acid side at various fixed concentrations of $(NH_4)_2SO_4$. In this way, $[NH_4^+]$ and $[NH_3]$ can be varied. If the pH's chosen are within the range at which k_d is constant, a linear graph will be obtained when $1/CP$ is plotted vs. the reciprocal of the true substrate, whereas a family of straight lines of common intercept and different slopes with changing pH's will be found with the reciprocal of the other species.

The results depicted in Figures 5 and 6 are congruent with NH_3 being the true substrate for the conversion of CO_2-P to CP at or near physiological pH. From the slope of the line of Figure 6, a value for $k_n = 8 \times 10^4\ M^{-1}\ s^{-1}$ can be calculated for the reaction of CO_2-P with NH_3.

Formation of an unstable phosphoenzyme with concomitant release of ADP in the absence of HCO_3^- and/or AG was tested. As shown in Figure 7, mixing stoichiometric amounts of the enzyme and ATP did not cause marked production of ADP and the presence of AG or HCO_3^- alone had no effect, whereas subsequent addition of both components caused an instantaneous and continued production of ADP. These findings support previous kinetic studies (Elliott and Tipton, 1974b) and indicate that if a phosphoenzyme were formed prior to reaction with HCO_3^- it should be stable in the absence of bicarbonate. Otherwise, decomposition of the phosphoenzyme should result in release of ADP before a new molecule of ATP reacts. Without added HCO_3^- Metzenberg et al. (1958) have reported with mitochondrial carbamoyl-phosphate synthetase ca. ¼ the (AG-dependent) ATPase activity of that measured in the presence of HCO_3^-. This activity probably reflects contaminating HCO_3^- in the solutions (no special precautions to exclude HCO_3^- were reported). In our experiments, we purged rigorously (see legend of Figure 7) all solutions including the concentrated enzyme. As illustrated, under these conditions there is very little ADP liberation (0.75 mol of ADP/mol of carbamoyl-phosphate synthetase × min which is a maximal figure due to the extreme difficulties in securing solutions absolutely free of HCO_3^- at neutral pH).

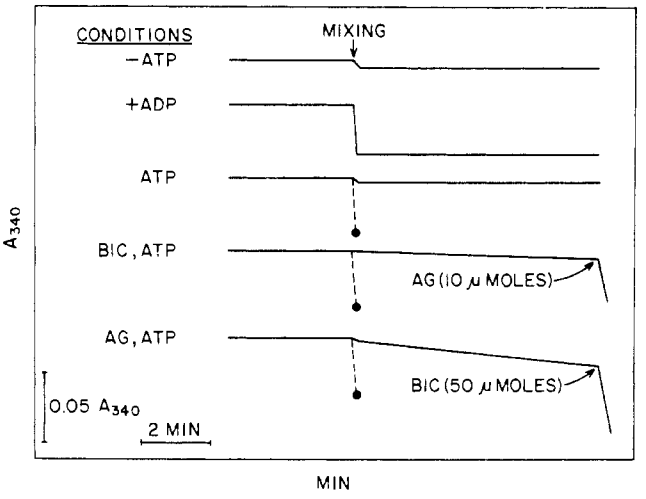


FIGURE 7: Changes in A_{340} on addition of ATP to substrate quantities of carbamoyl-phosphate synthetase. Double-sector cells (0.875-cm light path) were used. A chamber contained in 1 ml, 50 μmol of potassium Tris-maleate, pH 7.4, 11 nmol of carbamoyl-phosphate synthetase, and, when indicated, 10 nmol of ADP, 10 μmol of AG, and 50 μmol of $KHCO_3^-$. The other chamber contained in 1 ml, 50 μmol of potassium Tris-maleate, pH 7.4, 35 μmol of $MgSO_4$, 5.8 μmol of phosphoenolpyruvate (cyclohexylammonium salt), 11 μmol of mercaptoethanol, 0.55 μmol of NADH, 35 μg of lactic dehydrogenase, 100 μg of pyruvate kinase, and 1.1 μmol of ATP (except when so indicated). After temperature equilibration, the contents of both chambers were mixed. Approximately 15 s elapsed between the mixing and the first recording of A_{340}. The calculated values for a phosphoenzyme assuming 1 mol of ADP formed per mol of carbamoyl-phosphate synthetase are shown by the broken lines. All solutions were degassed at pH ~4 (10-min vacuum) and then neutralized with the calculated amounts of concentrated KOH under N_2. Pyruvate kinase and lactate dehydrogenase were then added. Lyophilized carbamoylphosphate synthetase was dissolved, under N_2, in buffer.

Discussion

This paper clearly demonstrates the formation of active CO_2 with frog liver carbamoyl-phosphate synthetase. Existence of active CO_2 was previously suggested by maximal protection against Nbs_2 inactivation of mitochondrial carbamoyl-phosphate synthetase with ATP, Mg^{2+}, K^+, HCO_3^-, and AG (Novoa and Grisolia, 1970). As already indicated, absence of ammonia is essential for the demonstration of CO_2-P; we solved this by preincubating the enzyme with ATP and HCO_3^- prior to the addition of $H^{14}CO_3^-$, thereby "removing" ammonia as CP. $^{14}CO_2$ exchange between $^{14}CO_2$-P and the small amount of nonradioactive CP present (due to the removal of ammonia by conversion to CP) was nil under the conditions used. This is shown by the low background values obtained when KOH was added before NH_3 (see Table I) and by the horizontal character of the plateaus of Figure 4 (which would have an upward slope if any exchange had been present).

It is generally accepted that an intermediate of the type studied in this paper would be very unstable, as reflected by the ATPase activity of carbamoyl-phosphate synthetase. Indeed, Guthöhrlein and Knappe (1969) calculated, on the basis of two active sites per enzyme molecule, a half-life ≥ 2 s for active CO_2 with rat liver mitochondrial carbamoyl-phosphate synthetase. However, under the usual conditions at which the ATPase activity of carbamoyl-phosphate synthetase is assayed, the accumulation of CO_2-P is ~3.4 times the amount of carbamoyl-phosphate synthetase (see Results). A half-life at 37 °C of ~2.8 s for CO_2-P can thus be obtained based on the ATPase activity of frog carbamoyl-phosphate synthetase (less than 7% of the rate of CP synthesis (Rubio and Grisolia, un-

published)), if 1 mol of ADP is produced per mol of CO_2-P decomposed. However, if 2 mol of ADP are produced, since likely 2 ATP molecules are used to form CO_2-P, the half-life of CO_2-P will be ~5.6 s.

When the rate of CO_2-P decomposition required to account for the ATPase activity of carbamoyl-phosphate synthetase is compared with the rate of disappearance of CO_2-P ($t_{1/2} \simeq$ 0.3 s; see Results), the latter is ca. tenfold faster. Therefore, CO_2-P disappearance can take place by mechanisms other than by the production of ADP and P_i, probably by reversal of CO_2-P synthesis at neutral pH's. Alternative possibilities, such as decomposition of CO_2-P to stable products other than ADP and P_i (for example, AMP and pyrophosphate), seem unlikely.

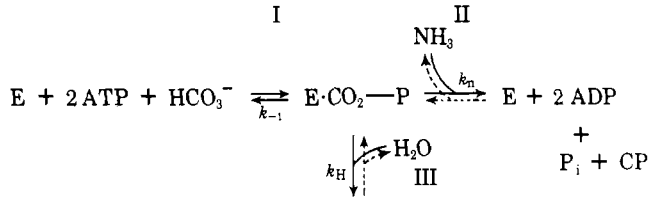
Sauers et al. (1975) have discussed anew carbonyl phosphate as an intermediate in CP synthesis (and in enzymatic carboxylations requiring biotin) to explain ^{18}O transfer from HCO_3^- to P_i (Jones and Spector, 1960) with carbamoyl-phosphate synthetase. They calculated a rate constant for carbonyl phosphate decomposition at 25 °C $\geq 10\ s^{-1}$ ($t_{1/2} \geq$ 0.069 s). This value is much greater than required to account for the ATPase activity of carbamoyl-phosphate synthetase.

When CO_2-P is produced in the absence of ammonia, it rapidly attains a steady-state level, which is dependent on the concentrations of ATP, HCO_3^-, and carbamoyl-phosphate synthetase. The faster rate of CO_2-P synthesis (equal to the rate of its disappearance at the steady state) than of ADP liberation by carbamoyl-phosphate synthetase (in the absence of ammonia) and the identity of the K_M^{ATP} for the ATPase and the ATP concentration at which half-maximal CO_2-P concentration is obtained (K_M^{ATP}) indicate that the rate of the ATPase activity of carbamoyl-phosphate synthetase is determined by the rate of ADP-releasing decomposition of CO_2-P.

Likely, CO_2-P is enzyme bound, because its maximal accumulation at the steady state is limited by the concentration of carbamoyl-phosphate synthetase. At infinite ATP and HCO_3^- concentrations, 4.1 mol of CO_2-P/mol carbamoyl-phosphate synthetase can be calculated (see Results) or, if bound, 4 active sites/320 000 D carbamoyl-phosphate synthetase dimer. While two catalytic sites per subunit seems unlikely, it may reflect the very large size of the individual peptidic chains of mitochondrial carbamoyl-phosphate synthetase (Marshall, 1976).

The reaction of CO_2-P with ammonia does not consume free ATP (see Table III) and thus both moles of this nucleotide are bound to the enzyme prior to reaction with ammonia. This supports the sequence reported by Elliott and Tipton (1974a). Therefore, the mechanism proposed by Jones (1976) and by Sauers et al. (1975) postulating formation of a carbamate-like intermediate before interaction of the enzyme with the second mole of ATP can be ruled out as presented if ammonia is used for formation of carbamate. However, it could be made consistent with our data if modified to include the binding of the second ATP molecule to the enzyme prior to reaction with NH_3: perhaps one ATP reacts to give the carbonyl phosphate anhydride, which then reacts with NH_3 to give carbamate and then carbamate is phosphorylated by another bound ATP to give CP, which dissociates from the enzyme first, followed by dissociation of two ADP and P_i.

A more direct mechanism involving two ATP molecules in the synthesis of CO_2-P congruent with the findings reported in this paper is:



$$E + HCO_3^- + 2\,ADP + 2\,P_i$$

or

$$E + HCO_3^- + ADP + ATP + P_i$$

The suggested mechanism is simpler, for it does not require separate sequences for the binding and for the reaction of the substrates, nor does it require delayed release of ADP and P_i to account for the kinetic and isotopic exchange data (Elliott and Tipton, 1974b; Guthöhrlein and Knappe, 1969).

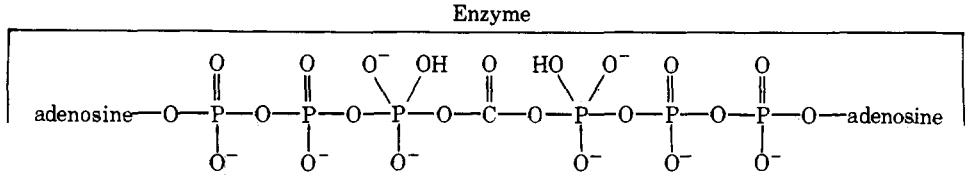
Reaction III must be essentially irreversible, since no ADP or P_i-ATP exchange has been detected with carbamoyl-phosphate synthetase in the absence of NH_3. The very slow rate of exchange when NH_3 is present indicates that reaction II, in the reverse direction, plays a very limited role in the mechanism. For simplicity, no attempt has been made to include AG, Mg^{2+}, and K^+ in the mechanism. While clearly these activators are needed to generate CO_2-P, their roles, if any, in reactions II and III are not known.

NH_3 (and not NH_4^+) seems to be the chemical species reacting with CO_2-P to produce CP. This is not surprising from the nucleophilic character of NH_3. Because NH_4^+ predominates at physiological pH's, Jones (1976) suggested that carbamoyl-phosphate synthetase could bind NH_4^+ and then remove a proton to produce NH_3 in situ. This does not seem to be the case, as shown by the experiments presented in Figures 5 and 6.

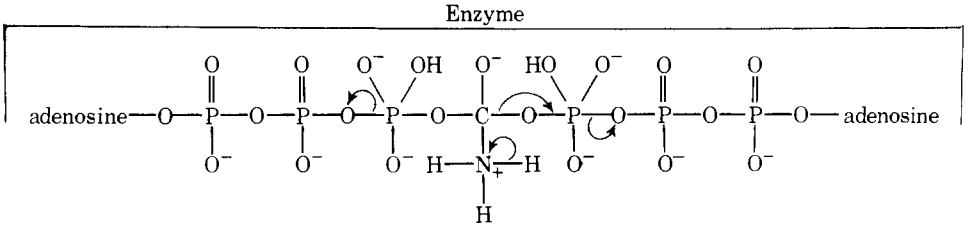
The values for k_n, k_H, and $k_{-1} = k_d - k_H$ in the preceeding scheme would be $8 \times 10^4\ M^{-1}\ s^{-1}$ (25 °C; see Results), 0.25 or $0.125\ s^{-1}$ (37 °C, one or two ADP molecules produced per molecule of CO_2-P decomposed; water included in the constant), and 2.05 or $2.17\ s^{-1}$ (37 °C), respectively.

It seems likely from these data that the rate of CP synthesis at saturation of NH_3 should approximate that of CO_2-P synthesis, since the pseudo-first-order constant for reaction II forward at saturation of NH_3 will have a value much greater than k_{-1} (and obviously than k_H).

While carbonyl phosphate anhydride can be postulated as a step in the mechanism involving carbamate synthesis, a structure of the following type would be in harmony with the mechanism presented above. Such a type of structure would be certainly unstable, but interaction with the protein perhaps would contribute to its stabilization.



The formation of CP would only occur on the subsequent addition of ammonia:



The above could explain the ATPase reaction; i.e., by direct reaction of the intermediate with H_2O, instead of NH_3, there will be production of ADP, P_i, and carbonyl phosphate. The latter, in turn, will decompose by decarboxylation (or hydrolysis).

By analogy a related mechanism may occur for biotin-dependent carboxylation reactions. However, in this case, a single ATP participates and, therefore, a single anhydride, i.e., rather than a double anhydride, may be visualized, as postulated by Kaziro et al. (1962) and Moss and Lane (1971). Therefore, pulse-label experiments with biotin-dependent enzymes should be carried out.

The fact that reaction of NH_3 with CO_2-P takes place in acetone suggests that the reaction does not require enzyme catalysis and that the main role of mitochondrial carbamoyl-phosphate synthetase is to synthesize CO_2-P, which would then react with NH_3 or with other compounds (NH_2NH_2, NH_2OH, H_2O) at a rate determined by the nucleophilic character of the attacking group. Steric and electrostatic factors would restrict the access of certain molecules (e.g., Tris, glycylglycine) to the active site. The role of carbamoyl-phosphate synthetase at this step would be to impose these restrictions, conferring a high degree of specificity to the reaction.

Finally, the possibility cannot be excluded that the active site becomes "frozen" by acetone in a proper conformation and, as such, catalyzes this partial reaction. If this is the case, it could provide a much needed model for evolutionary studies and for the search for a common ancestral enzyme. It should be noted that, although there are many examples of homologous enzymes, phosphoglyceromutases, aldolases, and carbamoyl-phosphate synthetases (and kinases) are, thus far, possibly the only known examples of analogue enzymes.

Note Added

After submission of this manuscript, an abstract appeared (Powers and Meister, 1976) reporting the isolation of a stable derivative of a carbonate phosphate anhydride with *E. coli* carbamoyl-phosphate synthetase. Unlike that formed by mitochondrial carbamoyl-phosphate synthetase, the activated CO_2 formed with *E. coli* carbamoyl-phosphate synthetase has a half-life of minutes, thus making possible its reaction with diazomethane and the isolation of a derivative. Since the alleged *E. coli* activated CO_2 is three orders of magnitude more stable than predicted for free carbonate phosphate anhydride (Sauers et al., 1975), it is reasonable to assume that it is bound to the enzyme, as is also likely with the mitochondrial carbamoyl-phosphate synthetase. Their marked difference in stability is puzzling and interesting for the reported half-life of the *E. coli* active CO_2 we have calculated to be at least two orders of magnitude higher than required if the activated CO_2 is enzyme bound, based on the bicarbonate-dependent ATPase activity of the *E. coli* carbamoyl-phosphate synthetase (Anderson and Meister, 1966).

Acknowledgments

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pH-Induced Changes in the Reactions Controlled by the Low- and High-Affinity Ca^{2+}-Binding Sites in Sarcoplasmic Reticulum†

Sergio Verjovski-Almeida‡ and Leopoldo de Meis*

ABSTRACT: The effect of pH on the Ca^{2+}-binding sites of high and low affinity, located respectively on the outer and inner surfaces of the sarcoplasmic reticulum membrane, was investigated using intact and leaky sarcoplasmic reticulum vesicles. With the use of intact vesicles, different pH profiles of membrane phosphorylation and rates of nucleoside triphosphate hydrolysis were obtained depending on the assay temperature, on the Ca^{2+} concentration, and on whether ATP or ITP was used as substrate. The different pH profiles were related to the amount of Ca^{2+} accumulated by the vesicles, i.e., to different degrees of saturation of the inner, low-affinity Ca^{2+}-binding site. With the use of leaky vesicles, the saturation of the two Ca^{2+}-binding sites could be controlled more precisely since the Ca^{2+} concentration on both sides of the membrane was equal to the Ca^{2+} concentration of the assay medium. Using leaky vesicles and measuring the rates of nucleotide hydrolysis, nucleotide–phosphate exchange and membrane phosphorylation by nucleotide as an indication of the degree of saturation of the Ca^{2+}-binding sites, we observed that the affinity of both the high- and low-affinity sites increased three to four orders of magnitude when the pH of the assay medium was increased from 6.1 to 8.65.

The Ca^{2+} accumulation by sarcoplasmic reticulum vesicles isolated from skeletal muscle is mediated by a membrane-bound NTPase[1] (Hasselbach, 1974; MacLennan and Holland, 1975). The reversibility of the Ca^{2+} pump, involving NTP synthesis and Ca^{2+} efflux, has been demonstrated (Barlogie et al., 1971; Makinose and Hasselbach, 1971; Hasselbach, 1974). Two types of Ca^{2+}-binding sites having different affinities appear to be involved in the forward and reverse reaction. When only the high-affinity site, located on the outer surface of the membrane, is saturated, the transport enzyme (E) is phosphorylated by NTP ($E \sim P$) and the NTPase activity is maximal (Hasselbach, 1974; de Meis and Carvalho, 1974; Ikemoto, 1974; Souza and de Meis, 1976). Different steady-state levels of phosphoenzyme are obtained depending on whether ATP or ITP is used (Souza and de Meis, 1976). This has been interpreted to mean that ATP besides acting as substrate could also activate the rate of $E \sim P$ hydrolysis (de Meis and de Mello, 1973; Froehlich and Taylor, 1975; Carvalho et al., 1976; Souza and de Meis, 1976).

When only the low-affinity Ca^{2+}-binding site, located in the inner surface of the membrane, is saturated, the enzyme is phosphorylated by P_i, the phosphate is transferred to ADP, and ATP is synthesized (Makinose, 1972; Yamada et al., 1972; de Meis, 1976; de Meis and Carvalho, 1976). The simultaneous binding of calcium to both low- and high-affinity sites inhibits the NTPase activity and activates a steady exchange between P_i and the γ-phosphate of NTP (de Meis and Carvalho, 1974; Ikemoto, 1974; de Meis and Sorenson, 1975; Carvalho et al., 1976). This exchange is the result of the transport enzyme operating simultaneously forward (NTP hydrolysis) and backward (NTP synthesis from NDP and P_i), where part of the energy derived from the NTP hydrolysis seems to be conserved by the system for the synthesis of a new NTP molecule (de Meis and Carvalho, 1974; Carvalho et al., 1976).

In this paper, we attempted to modify the affinity of the two Ca^{2+}-binding sites by varying the pH of the assay medium (Meissner, 1973; Verjovski-Almeida and de Meis, 1975). The

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‡ Recipient of a fellowship from CNPq, Brasil.

[1] Abbreviations used: NTPase, nucleoside 5′-triphosphatase; NTP, nucleoside 5′-triphosphate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; SE, standard error.