Carbamoyl-Phosphate Synthetase II of the Mammalian CAD Protein: Kinetic Mechanism and Elucidation of Reaction Intermediates by Positional Isotope Exchange†

Thomas D. Meek,* William E. Karsten, and Charles W. DeBrosse

Departments of Medicinal Chemistry and Analytical, Physical, and Structural Chemistry, Smith Kline & French Laboratories, Swedeland, Pennsylvania 19479

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ABSTRACT: The kinetic mechanism of carbamoyl-phosphate synthetase II from Syrian hamster kidney cells has been determined at pH 7.2 and 37 °C. Initial velocity, product inhibition, and dead-end inhibition studies of both the biosynthetic and bicarbonate-dependent adenosinetriphosphatase (ATPase) reactions are consistent with a partially random sequential mechanism in which the ordered addition of MgATP, HCO_3^-, and glutamine is followed by the ordered release of glutamate and P_i. Subsequently, the binding of a second MgATP is followed by the release of MgADP, which precedes the random release of carbamoyl phosphate and a second MgADP. Carbamoyl-phosphate synthetase II catalyzes $\beta\gamma$-bridge:β-nonbridge positional oxygen exchange of [γ-^{18}O]ATP in both the ATPase and biosynthetic reactions. Negligible exchange is observed in the strict absence of HCO_3^- (and glutamine or NH_4^+). The ratio of moles of MgATP exchanged to moles of MgATP hydrolyzed (v_{ex}/v_{cat}) is 0.62 for the ATPase reaction, and it is 0.39 and 0.16 for the biosynthetic reaction in the presence of high levels of glutamine and NH_4^+, respectively. The observed positional isotope exchange is suppressed but not eliminated at nearly saturating concentrations of either glutamine or NH_4^+, suggesting that this residual exchange results from either the facile reversal of an E–MgADP–carboxyphosphate–Gln(NH_4^+) complex or exchange within an E–MgADP–carbamoyl phosphate–MgADP complex, or both. In the ^{31}P NMR spectra of the exchanged [γ-^{18}O]ATP, the distribution patterns of ^{16}O in the γ-phosphorus resonances in all samples reflect an exchange mechanism in which a rotationally unhindered molecule of [$^{18}O_3$,^{16}O]P_i does not readily participate. These results suggest that the formation of carbamate from MgATP, HCO_3^-, and glutamine proceeds via a stepwise, not concerted mechanism, involving at least one kinetically competent covalent intermediate, such as carboxyphosphate.

The carbamoyl-phosphate synthetases (CPS;[1] EC 2.7.2.5) catalyze the ATP-dependent formation of carbamoyl phosphate (eq 1), a metabolite of the urea cycle and a precursor

$$2\text{MgATP} + HCO_3^- + \text{Gln}\ (NH_3) \rightarrow 2\text{MgADP} + \text{carbamoyl-P} + P_i + \text{Glu} \quad (1)$$

of pyrimidine nucleotides formed via the de novo biosynthetic pathway. In the absence of ammonia or glutamine, a bicarbonate-dependent ATPase reaction has been demonstrated (eq 2) (Anderson & Meister, 1966).

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

Several different types of carbamoyl-phosphate synthetase are found, depending on the source of the enzyme and its cellular function. The bacterial CPS is a dimer of nonidentical subunits (M_r ~130 000 and 48 000) that may use either glutamine or, less efficiently, ammonia as a nitrogen source. Its carbamoyl phosphate product is used in both the urea cycle and pyrimidine biosynthesis.

In mammals, separate enzymes exist for these two metabolic roles. Carbamoyl-phosphate synthetase I, a monomeric protein obtained from liver mitochondria (M_r ~160 000), has considerable sequence homology with the bacterial enzyme (Nyunoya et al., 1985), but it utilizes only ammonia as its nitrogen source and functions solely in the urea cycle. Carbamoyl-phosphate synthetase II, on the other hand, is found in the cytosol of mammalian cells, and its carbamoyl phosphate product is the first chemical intermediate of UMP biosynthesis. Its ability to use both glutamine and ammonia is similar to that of the bacterial CPS. Undoubtedly, segregation of these two carbamoyl-phosphate synthetases affords the mammalian cell more stringent individual control of these two metabolic pathways.

The structure of carbamoyl-phosphate synthetase II is remarkably different from that of either the bacterial enzyme or the mitochondrial enzyme. The synthetase is one of three enzymatic activities found within the trifunctional protein CAD, an oligomeric protein consisting of identical chimeric subunits (M_r ~210 000) (Shoaf & Jones, 1973; Mori & Tatibana, 1975; Coleman et al., 1977). In addition to CPS II, each subunit contains the aspartate transcarbamoylase and dihydroorotase activities of de novo pyrimidine biosynthesis. Presumably, by expressing these three enzymes as a single gene product, the organism exerts coordinate regulation of this

† A preliminary report of this work has been presented (Meek et al., 1986).

* Correspondence should be addressed to this author.

[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; Ap_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate; ATCase, aspartate transcarbamoylase; ATPase, adenosinetriphosphatase; CAD, trifunctional protein containing the first three enzymatic activities of de novo pyrimidine biosynthesis; carbamoyl-P, carbamoyl phosphate; CPS, carbamoyl-phosphate synthetase; Da, dalton; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; PALA, *N*-(phosphonoacetyl)-L-aspartate; PEP, phosphoenolpyruvate; PIX, positional isotope exchange; Tris, tris(hydroxymethyl)aminomethane; UMP, uridine 5′-monophosphate.

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pathway. CPS II occurs as a discrete domain of the 210-kDa polypeptide (Mally et al., 1981), and unlike the bacterial enzyme, its glutamine amidotransferase activity appears to be housed within the same polypeptide chain that also binds MgATP and bicarbonate.

An increase in carbamoyl-phosphate synthetase II activity attends the neoplastic transformation of some cells. Compared with corresponding normal tissue, the six enzymes of de novo UMP biosynthesis display greatly increased activities in a broad spectrum of mammalian tumors (Weber, 1983). These enhanced activities in transformed cells reflect a greater dependence on nucleic acid precursors and suggest that blockade of this pathway may prove more toxic to neoplastic tissues than to their normal counterparts. In addition, under conditions of pyrimidine nucleotide deprivation, normal cells, unlike transformed ones, are apparently capable of arresting their growth reversibly in phase G_0 of the cell cycle and as such are afforded protection to these adverse conditions (Pardee et al., 1978). Presumably for these reasons, *N*-(phosphonoacetyl)-L-aspartate (PALA), a powerful inhibitor of the second enzyme of UMP biosynthesis, aspartate transcarbamoylase, has proven to be curative toward various murine tumors (Johnson et al., 1976). Unfortunately, PALA has been considerably less effective in the treatment of human cancers.

As carbamoyl-phosphate synthetase II is both the first and rate-limiting enzyme of UMP biosynthesis, it is the enzyme target of choice for chemotherapeutic disruption of pyrimidine biosynthesis. As such, we sought to uncover details of the reaction mechanism of this enzyme that could lead to the design of specific inhibitors. The kinetic mechanism of an enzyme, that is, the order of addition of substrates and release of products on the enzyme surface, constitutes the first level of understanding of its actual chemical mechanism. From this information one may greatly minimize the number of possible chemical mechanisms and propose structures for the enzyme-bound reaction intermediates as suggested by the sequence of substrate interactions. Previous steady-state kinetic studies of the carbamoyl-phosphate synthetases from *Escherichia coli* (Raushel et al., 1978) and bovine liver mitochondria (Elliott & Tipton, 1974a,b) indicate that although these enzymes possess common structural features, they operate via different kinetic mechanisms. Given this and the structural disparity between CPS II and these enzymes, it is possible that CPS II makes use of a third mechanism for the carbamoyl-phosphate synthetases involving unique enzyme-bound reaction intermediates.

In this report, we present the kinetic mechanism of the CPS II of Syrian hamster kidney cells, as elucidated from a steady-state analysis of both the biosynthetic (eq 1) and ATPase (eq 2) reactions catalyzed by the enzyme. In addition, by employing the positional isotope exchange technique of Midelfort and Rose (1976), we have obtained evidence that carboxyphosphate is a kinetically competent intermediate of the CPS II catalyzed reactions.

Materials and Methods

Carbamoyl-phosphate synthetase II was purified from 165-23 cells, a line of SV40-transformed Syrian hamster kidney cells that are highly resistant to *N*-(phosphonoacetyl)-L-aspartate (Kempe et al., 1976). The cells (a generous gift of Dr. David R. Evans of Wayne State University) were continuously grown and harvested as described (Kempe et al., 1976). The enzyme was isolated as the trifunctional protein, CAD, by the method of Coleman et al. (1977) with several minor modifications. A mixture of protease inhibitors (Meek et al., 1985) was included in the lysis buffer to minimize proteolytic "nicking" of the CAD protein. Buffer A, which was used as the eluant in the gel filtration step, consisted of 20 mM Hepes (pH 7.4, 0 °C), 4 mM aspartate, 0.1 mM EDTA, 1 mM DTT, 30% (v/v) dimethyl sulfoxide, and 5% (w/v) glycerol. Fractions from the gel filtration step that contained nonhomogeneous protein were subjected to additional chromatography and concentration on a small column (0.5 mL) of DEAE-Sepharose CL-6B (Pharmacia); after washing the column with buffer A (pH 7.4), CPS II activity was eluted with a minimum volume of buffer A containing 0.3 M KCl. $NaDodSO_4$–polyacrylamide gel electrophoresis (6% polyacrylamide) indicated that the purity of the CAD protein exceeded 95% and that its subunit molecular weight was 209 000, containing very little of the M_r 190 000 proteolytic fragment reported by Coleman et al. (1979).

Typically, the purified CPS II fractions contained a specific activity of 0.06–0.08 nmol of carbamoyl phosphate/(min·μg) when assayed by the procedure of Coleman et al. (1977). The purified enzyme was routinely stored in buffer A at −80 °C, retaining most of its activity for as long as 8 months. Protein concentrations were determined by the method of Bradford (1976).

[^{14}C]Sodium carbonate (50–55 mCi/mmol) was purchased from Amersham or New England Nuclear and was generally stored and used as a 1.0 M solution of [^{14}C]$KHCO_3$ (0.45 mCi/mmol) in 0.1 M Tris-HCl (pH 8.5). Phosphoenolpyruvate carboxylase (wheat) and 5′-adenylyl imidodiphosphate (AMP-PNP) were obtained from Boehringer Mannheim Biochemicals, and other chemicals were purchased from Sigma. PEP carboxylase was purified before use by size exclusion high-performance liquid chromatography [column: Bio-Sil TSK-400 (Bio-Rad); 300 × 7.5 mm], and protein was eluted with 50 mM KH_2PO_4–0.3 M KCl (pH 6.5) at a flow rate of 0.5 mL/min.

Preparation of [γ-^{18}*O*]*ATP*. [$^{18}O_4$]KH_2PO_4 was prepared from PCl_5 and $H_2{}^{18}O$ (Stohler/KOR Isotopes; 99% ^{18}O) by the method of Hackney et al. (1980), and the product consisted of 92% [$^{18}O_4$]KH_2PO_4 as shown by ^{31}P NMR. [γ-$^{18}O_4$]ATP was then prepared by incubating 1.0 mmol of the mono(tri-*n*-butylammonium) salt of [$^{18}O_4$]phosphate (Moffatt, 1964) with 0.72 mmol of ADP-morpholidate (Wehrli et al., 1965) in 3.0 mL of rigorously dried dimethyl sulfoxide at 35 °C for 20 h. The product [γ-^{18}O]ATP (0.68 mmol, 93% as determined by hexokinase/glucose-6-phosphate dehydrogenase assay) was purified by ion-exchange chromatography as described (Wehrli et al., 1965) and recovered as its lyophilized triethylammonium salt.

Enzyme Assays. Initial rates of the reactions catalyzed by carbamoyl-phosphate synthetase II were determined at 37 °C by using radiometric, colorimetric, and spectrophotometric assay methods. All assays were conducted at pH 7.2, and $MgCl_2$ was held at concentrations that were 5–10 mM in excess of that of either ATP or ADP. The radiometric assay method was a modification of the procedure of Coleman et al. (1977) and was used whenever glutamine was the variable substrate or when MgADP was added. Radiometric assays were performed in a 0.5-mL total volume containing 50 mM Hepes (pH 7.2), 100 mM KCl, 1 mM DTT, 10% (w/v) glycerol (buffer B), 15 mM aspartate, 5–20 μg of CPS II, and various concentrations of substrates and products. Following incubation at 37 °C for a minimum of 7 min, reaction was initiated by the addition of either enzyme or an aliquot of 1.0 M [^{14}C]$KHCO_3$ (200–1000 dpm/nmol). The endogenous aspartate transcarbamoylase activity of the CAD protein was sufficient to convert the [^{14}C]carbamoyl phosphate formed to

the stable, nonvolatile [^{14}C]-*N*-carbamoylaspartate. After 20 min, reaction was quenched with 0.05 mL of 50% acetic acid, the remaining [^{14}C]$KHCO_3$ in the reaction mixture was evaporated on a heating plate, and the [^{14}C]-*N*-carbamoylaspartate in the residue was determined by scintillation counting. Blank samples containing either no enzyme or no glutamine yielded identical results, and the assay displayed a linear time course for at least 60 min.

CPS II activity was also measured spectrophotometrically by using the pyruvate kinase/lactate dehydrogenase coupling system. Initial rates were followed at 340 nm on a Hewlett-Packard 8450 spectrophotometer equipped with a Hewlett-Packard 85A computer for data analysis, and reaction temperature was maintained at 37 ± 0.5 °C with a Lauda RM6 bath which circulated water through jacketed cuvettes connected in series. Each 1-mL reaction mixture contained buffer B (pH 7.2), 33 μg each of pyruvate kinase and lactate dehydrogenase, 0.2 mM NADH, 1 mM PEP, 5–20 μg of CPS II, and various concentrations of substrates, products, and inhibitors. After incubation at 37 °C for at least 7 min, reaction was initiated by the addition of either glutamine or enzyme. A linear time course was observed for at least 60 min.

Similarly, the ATPase activity of CPS II was measured in the absence of added glutamine. Using the method of Levitski (1970), the concentration of endogenous ammonia in such reaction mixtures was shown to be ≤2 μM. By omission of $KHCO_3$ from the reaction mixture or by addition of Ap_5A to the reaction mixture, initial rates were found to be identical with that of NADH oxidation at 37 °C, indicating that no appreciable ATPase activity existed in the absence of added $KHCO_3$. In the presence of MgADP, initial rates of the ATPase reaction were measured by the colorimetric determination of inorganic phosphate. Briefly, 0.5-mL reaction mixtures were quenched after 1 h with 0.1 mL of acid-washed Norit (9 mg/mL) in 0.2 M HCl. The charcoal was removed by centrifugation, and a 0.3-mL aliquot of the supernatant was incubated with 0.7 mL of the ammonium molybdate–ascorbic acid reagent of Ames (1968) for 20 min at 45 °C, after which time absorbance was read at 790 nm. Samples were kept in ice following quenching to minimize P_i formation due to acid-catalyzed hydrolysis of ATP/ADP, and both nucleotides were added to all of the quenched samples to a uniform concentration in order to "normalize" the adventitious formation of inorganic phosphate.

Positional Isotope Exchange in [γ-^{18}O]ATP. The extent of βγ-bridge:β-nonbridge positional oxygen exchange in [γ-^{18}O]ATP was determined from the ^{31}P NMR signals of the γ-[$^{18}O_4$]-P and γ-[$^{18}O_3$]-P species, owing to a chemical shift difference of ~0.02 ppm (Cohn & Hu, 1978). In a 1.0-mL volume, carbamoyl-phosphate synthetase II was incubated in buffer B (pH 7.2) with 12 mM $MgCl_2$, 2 mM [γ-^{18}O]ATP, and various concentrations of the other substrates at 37 °C for 180 min. ADP formation was quantified at the end point by using the pyruvate kinase/lactate dehydrogenase coupling system. Bicarbonate-free samples were obtained by bubbling a stream of nitrogen through buffer prior to addition of enzyme and by adding 66 μg of purified PEP carboxylase and 4.5 mM PEP to separate solutions containing [γ-^{18}O]ATP and CPS II. The enzymatic removal of endogenous bicarbonate was monitored at 340 nm by coupling the product oxaloacetate to the NADH-dependent malate dehydrogenase reaction. An aliquot of the mixture was added to a 1.0-mL assay solution containing 5 mM $MgCl_2$, 1 unit of malate dehydrogenase, and 0.2 mM NADH, and the separate solutions containing [γ-^{18}O]ATP and CPS II were combined after bicarbonate concentrations had fallen to ≤5 μM.

The samples were quenched by adding 0.4 mL of 0.2 M Na_2EDTA–0.2 M Tris (pH 10.0), followed by vigorous vortexing. Protein was precipitated with a few drops of $CHCl_3$ and removed by centrifugation. The samples were made 30% in D_2O, and ^{31}P NMR spectra were measured by using a Bruker Instruments WM-360 spectrometer operating at 145.80 MHz. Typically, spectra were measured by using 16K data points, a sweep width of 3600 Hz, a pulse of 15 μs (54°), and a relaxation delay of 2 s. The samples were measured by using a 10-mm probe, regulated at 23 °C, and approximately 5000 scans were averaged to obtain an adequate signal-to-noise ratio. Prior to Fourier transformation, the resolution was enhanced by applying a shifted sine bell (30°) apodization over the first 8K data points.

Data Analysis. The kinetic data were analyzed by using the Fortran programs of Cleland (1967). Double-reciprocal plots were fitted to eq 3 or 4 for data describing a normal

$$v = VA/(K + A) \tag{3}$$

$$\log v = \log \frac{V(dA + A^2)}{c + bA + A^2} \tag{4}$$

hyperbolic or 2/1 function, respectively. In eq 4, $b = K_{a1} + K_{a2}$, $c = K_{a1}K_{a2}$, and $d = V_1K_{a2} + V_2K_{a1}$. Initial velocity data yielding apparently intersecting patterns were fitted to eq 5, parallel patterns to eq 6, and patterns that were intersecting on the $1/v$ axis to eq 7. Patterns conforming to competitive,

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \tag{5}$$

$$v = \frac{VAB}{K_aB + K_bA + AB} \tag{6}$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + AB} \tag{7}$$

noncompetitive, or uncompetitive inhibition were fitted to eq 8, 9, or 10, respectively. When the type of pattern was in

$$v = \frac{VA}{K[1 + (I/K_{is})] + A} \tag{8}$$

$$v = \frac{VA}{K[1 + (I/K_{is})] + A[1 + (I/K_{ii})]} \tag{9}$$

$$v = \frac{VA}{K + A[1 + (I/K_{ii})]} \tag{10}$$

doubt for an experiment, the data were fitted to both possible equations and a comparison of the resulting σ values (square root of the average residual least square) was made to determine the best fit. In no case did a set of data fit two equations equally well. The nomenclature used is that of Cleland (1963).

Results

Initial Velocity Data with Variable MgATP. As with the bacterial (Raushel et al., 1978) and beef liver (Elliott & Tipton, 1974a) enzymes, carbamoyl-phosphate synthetase II requires for its activity free magnesium ion in excess of that which is complexed as MgATP. At several fixed concentrations of MgATP (0.5–5.0 mM), maximal initial velocities of the CPS II catalyzed biosynthetic reaction were observed when the total concentration of Mg^{2+} was held between 3 and 5 mM in excess of that of the nucleotide substrate. Significant inhibition was observed when free Mg^{2+} exceeded 10 mM. As

Table I: Initial Velocity Patterns[a]

varied substrate	fixed substrate	pattern type	apparent Michaelis constants (mM) MgATP	HCO_3^-	Gln
MgATP vs. HCO_3^-	Gln, 0.03 mM	parallel	1.5 ± 0.2	3.4 ± 0.4	
MgATP vs. HCO_3^-	Gln, 3.3 mM	parallel	1.8 ± 0.3	8.6 ± 0.6	
MgATP vs. HCO_3^-		intersecting	0.19 ± 0.02	0.65 ± 0.09	
MgATP vs. Gln	HCO_3^-, 10 mM	parallel	0.8 ± 0.4		0.011 ± 0.002
HCO_3^- vs. Gln	MgATP, 2 mM	intersecting, equilibrium ordered		12 ± 4[b]	0.039 ± 0.007

[a] At pH 7.2, 37 °C, 100 mM KCl, and 5–10 mM excess $MgCl_2$. [b] The apparent dissociation constant.

Table II: Product Inhibition Patterns[a]

variable substrate	inhibitor	fixed substrates	inhibition pattern	K_{is} (mM)	K_{ii} (mM)
MgATP	MgADP	HCO_3^-, 10 mM; Gln, 3.3 mM	NC	1.3 ± 0.2	6 ± 1
MgATP	MgADP	HCO_3^-, 20 mM	NC	0.34 ± 0.07	5 ± 3
HCO_3^-	MgADP	MgATP, 2 mM; Gln, 0.03 mM	NC	0.7 ± 0.1	4.6 ± 0.4
HCO_3^-	MgADP	MgATP, 50 mM; Gln, 0.03 mM	UC		27 ± 5
HCO_3^-	MgADP	MgATP, 0.5 mM	NC	0.6 ± 0.3	0.9 ± 0.4
Gln	MgADP	MgATP, 2 mM; HCO_3^-, 10 mM	NC	5 ± 2	3 ± 1
Gln	MgADP	MgATP, 50 mM; HCO_3^-, 10 mM	UC		11 ± 2
Gln	MgADP	MgATP, 2 mM; HCO_3^-, 100 mM	UC		4.8 ± 0.8
MgATP	phosphate	HCO_3^-, 10 mM; Gln, 0.03 mM	NC	13 ± 4	170 ± 23
Gln	phosphate	MgATP, 2 mM; HCO_3^-, 10 mM	UC		69 ± 9
MgATP	Glu	HCO_3^-, 10 mM; Gln, 0.03 mM	UC		89 ± 20
HCO_3^-	Glu	MgATP, 2 mM; Gln, 0.03 mM	NC	12 ± 7	22 ± 5
Gln	Glu	MgATP, 2 mM; HCO_3^-, 10 mM	NC	9 ± 2	17 ± 2
MgATP	carbamoyl-P	HCO_3^-, 10 mM; Gln, 0.03 mM	UC		1.6 ± 0.1
HCO_3^-	carbamoyl-P	MgATP, 2 mM; Gln, 0.03 mM	NC	2.1 ± 0.4	13 ± 3
Gln	carbamoyl-P	MgATP, 2 mM; HCO_3^-, 10 mM	NC	2.4 ± 0.4	3.7 ± 0.3
MgATP	carbamoyl-P	HCO_3^-, 2 mM	C	1.5 ± 0.3	

[a] At pH 7.2, 37 °C, 100 mM KCl, and 5–10 mM excess $MgCl_2$. C, competitive inhibition; NC, noncompetitive inhibition; UC, uncompetitive inhibition; K_{is}, apparent slope inhibition constant; K_{ii}, apparent intercept inhibition constant.

such, all kinetic studies were conducted with Mg^{2+} concentrations 5–10 mM in excess of those of ATP and ADP.

For the biosynthetic reaction, double-reciprocal plots of $1/v$ vs. 1/[MgATP] at either saturating or nonsaturating concentrations of both HCO_3^- and glutamine were concave upward. The data conformed to a 2/1 function (eq 4), and at fixed levels of 0.133 M HCO_3^- and 3.3 mM glutamine, the resulting kinetic constants were $b = 4.7 \pm 1.5$ mM, $c = 2.2 \pm 0.6$, $d = 0.02 \pm 0.02$, $K_{MgATP,1} = 0.5 \pm 0.3$ mM, $K_{MgATP,2} = 4.2 \pm 1.5$ mM, and $V = 0.18 \pm 0.015$ nmol/(min·μg). Lyons and Christopherson (1985) observed similar results with CPS II and attributed this kinetic behavior to a positive homotropic interaction among the individual active sites of the oligomer for the binding of MgATP. Accordingly, we conducted all initial velocity and inhibition studies of the biosynthetic reaction with varied MgATP held at high concentrations (1–15 mM), at the linear asymptote of the curvilinear double-reciprocal plots.

In contrast, a plot of $1/v$ vs. 1/[MgATP] for the CPS II catalyzed ATPase reaction (glutamine absent) appeared to be linear over a similar range of MgATP concentrations. A fit of these data to eq 3 gave $K_{MgATP} = 0.17 \pm 0.016$ mM and $V = 0.072 \pm 0.0016$ nmol/(min·μg). As such, the positive cooperative binding of MgATP appears to be solely a property of the biosynthetic reaction and may reflect an interaction of enzyme and substrate that is kinetically distinct from that of the ATPase reaction.

Initial Velocity Patterns. Initial velocity data for the biosynthetic and ATPase reactions of CPS II are presented in Table I. The kinetic constants listed were obtained from fitting of the data to eq 5, 6, or 7. Double-reciprocal plots of HCO_3^- vs. MgATP at both 0.03 and 3.3 mM glutamine and glutamine vs. MgATP at 10 mM HCO_3^- all resulted in parallel patterns. At saturating glutamine, $K_{MgATP} = 1.8 \pm 0.3$ mM and $K_{bicarbonate} = 8.6 \pm 0.6$ mM. However, HCO_3^- vs. MgATP was intersecting for the ATPase reaction, and the apparent Michaelis constants ($K_{MgATP} = 0.19 \pm 0.02$ mM and $K_{bicarbonate} = 0.65 \pm 0.09$ mM) were considerably lower in the absence of glutamine. With identical, fresh preparations of enzyme, apparent V_{max}/E_t(ATPase) = 0.075 nmol of MgADP/(min·μg), and from a pattern of HCO_3^- vs. MgATP at 3.3 mM glutamine, apparent V_{max}/E_t(biosynthetic) = 0.15 nmol of carbamoyl phosphate/(min·μg).[2] It was found that the V_{max}(ATPase)/V_{max}(biosynthetic) ratio became greater than 0.5 following prolonged storage of the enzyme in buffer A at −80 °C, suggesting that its glutaminase activity becomes impaired in this milieu. HCO_3^- vs. glutamine at 2 mM MgATP resulted in a pattern that intersected on the $1/v$ axis, and the reported kinetic constants were obtained by fitting the data to eq 7 as glutamine vs. HCO_3^-. By use of the hexokinase/glucose-6-phosphate dehydrogenase coupling assay system as described (Meek & Villafranca, 1980), the CPS II catalyzed formation of MgATP at 50 mM phosphate, 50 mM glutamate, 10 mM MgADP, and 10 mM carbamoyl phosphate was found to be 1.3% of the rate of the forward biosynthetic reaction measured at 10 mM MgATP, 10 mM HCO_3^-, and 3.3 mM glutamine. Under appropriate conditions, no reversal of the ATPase reaction could be detected.

Product Inhibition Patterns. Results of product inhibition studies for both the biosynthetic and ATPase reactions are presented in Table II. All patterns displayed linear inhibition. For the biosynthetic reaction in which the fixed substrates were held at subsaturating concentrations, MgADP effected non-

[2] By comparison of V_{max} values from kinetic patterns of HCO_3^- vs. MgATP at 3.3 mM Gln obtained from both the radiometric and spectrophotometric assays using identical fresh preparations of enzyme, it was found that 1.9 mol of MgADP (NAD^+) was formed for each 1 mol of [^{14}C]carbamoyl phosphate produced. This indicates that with fresh CPS II the ATPase activity is negligible in the presence of saturating glutamine. When the spectrophotometric assay was used with aged enzyme, the ATPase rate was always subtracted from the biosynthetic rate by adding glutamine last to initiate the reaction mixture.

Table III: Positional Isotope Exchange of [γ-$^{18}O_4$]ATP Catalyzed by CPS II[a]

expt	additions	CPS II (mg)	percentage of γ-[$^{18}O_4$]-P[b]	fraction of exchange reaction (F)[c]	fraction of chemical reaction	v_{ex} [nmol/(min·mg)][d]	v_{ex}/v_{cat}[e]
1		0.0	64	0.0			
2	HCO_3^-, 5 mM	0.45	48	0.37	0.52	8.1	0.62
3	HCO_3^-, 5 mM; glutamine, 1 mM	0.45	50	0.32	0.63	6.0	0.39
4	HCO_3^-, 5 mM; NH_4^+, 100 mM	0.15	56	0.13	0.58	6.7	0.16
5	HCO_3^-, 5 mM; NH_4^+, 100 mM; aspartate, 5 mM; ATCase, 0.5 unit	0.15	57	0.11	0.50	6.2	0.17
6	PEP, 4.5 mM; PEP carboxylase, 0.05 unit	0.0	58	0.16			
7	PEP, 4.5 mM; PEP carboxylase, 0.05 unit	0.65	55	0.19		0.52[f]	

[a] At pH 7.2, 37 °C, and t = 180 min; 50 mM Hepes, 100 mM KCl, 12 mM $MgCl_2$, 1 mM DTT, 5% (w/v) glycerol, and 2 mM [γ-$^{18}O_4$]ATP. [b] The averaged values of the integrals: γ-[$^{18}O_4$]-P/(γ-[$^{18}O_4$]-P + γ-[$^{18}O_3$,^{16}O]-P + γ-[$^{18}O_2$,$^{16}O_2$]-P + γ-[$^{16}O_4$]-P). [c] $F = ([\gamma\text{-}^{18}O_4]_t - [\gamma\text{-}^{18}O_4]_0)/([\gamma\text{-}^{18}O_4]_{eq} - [\gamma\text{-}^{18}O_4]_0)$, where [$\gamma$-$^{18}O_4$] at t_0 and t_{eq} are 0.64 and 0.21, respectively. [d] $v_{ex} = [X/\ln(1 - X)](ATP_0/t)[\ln(1 - F)]$, where X = the percentage of ATP hydrolyzed during the course of the exchange reaction (Litwin & Wimmer, 1979). [e] v_{ex}/v_{cat} = micromoles of ATP exchanged per micromole of ADP formed in 180 min. [f] v_{ex} for experiment 7 is calculated from F(expt 7) − F(expt 6) = 0.03.

competitive inhibition vs. MgATP, HCO_3^-, and glutamine. However, at 50 mM MgATP, MgADP vs. HCO_3^- was uncompetitive. Similarly, MgADP vs. glutamine was uncompetitive at saturating levels of either MgATP (50 mM) or HCO_3^- (100 mM). For the ATPase reaction, MgADP was a noncompetitive inhibitor vs. either MgATP or HCO_3^-, and K_{is}, but not K_{ii}, was substantially lower in these patterns than in the corresponding patterns of the biosynthetic reaction. Replots of the slopes and intercepts of individual lines vs. MgADP concentration from any of the above patterns revealed no apparent parabolism. Phosphate, a poor product inhibitor of the biosynthetic reaction, effected noncompetitive inhibition vs. MgATP (K_{is} = 13 ± 4 mM; K_{ii} = 170 ± 23 mM) and uncompetitive inhibition vs. glutamine at subsaturating fixed levels of MgATP and HCO_3^-. Both glutamate and carbamoyl phosphate were uncompetitive inhibitors vs. MgATP and noncompetitive inhibitors vs. HCO_3^- and glutamine. In the absence of glutamine, however, the inhibition pattern of carbamoyl phosphate vs. MgATP was competitive.

Dead-End Inhibition Patterns. The ATP analogue AMP-PNP was resistant to CPS II catalyzed hydrolysis and, as such, was used as a dead-end inhibitor of the biosynthetic and ATPase reactions. All patterns displayed linear inhibition. AMP-PNP was a competitive inhibitor vs. MgATP in both the biosynthetic and ATPase reactions in which K_{is} = 6.5 ± 0.7 mM and 3.6 ± 0.6 mM, respectively. While it was an uncompetitive inhibitor vs. both HCO_3^- and glutamine in the biosynthetic reaction, AMP-PNP was a noncompetitive inhibitor vs. HCO_3^- under conditions of the ATPase reaction.

Positional Isotope Exchange of [γ-^{18}O]ATP. Studies of the CPS II catalyzed $\beta\gamma$-bridge:β-nonbridge positional oxygen exchange of [γ-^{18}O]ATP are summarized in Table III. Due to the 0.02-ppm difference in chemical shift (~3 Hz) in the ^{31}P NMR spectrum of a phosphate resonance when one of its atoms of ^{16}O is substituted by ^{18}O (Cohn & Hu, 1978), the extent of positional oxygen exchange could be directly determined in the recovered [γ-^{18}O]ATP by comparing the average integrals of the γ-[$^{18}O_4$]-P and γ-[$^{18}O_3$,^{16}O]-P resonances. The substitution pattern of ^{18}O atoms in the synthetic [γ-^{18}O]ATP is shown in the ^{31}P NMR spectrum in Figure 1A. Before or after incubation at 37 °C for 180 min under experimental conditions (experiment 1), ^{18}O labeling of the γ-phosphorus atom was found to be as follows: $^{18}O_4$, 63.5%; $^{18}O_3{}^{16}O$, 25.7%; $^{18}O_2{}^{16}O_2$, 5.6%; $^{18}O^{16}O_3$, not detectable; and $^{16}O_4$, 5.2%. With the inclusion of enzyme and HCO_3^- (experiment 2; Figure 1B) hydrolysis of ATP proceeded to about 50% with an accompanying diminution in the γ-[$^{18}O_4$]-P peak of the resynthesized ATP (48% of the total species), indicating that considerable positional oxygen exchange had occurred.

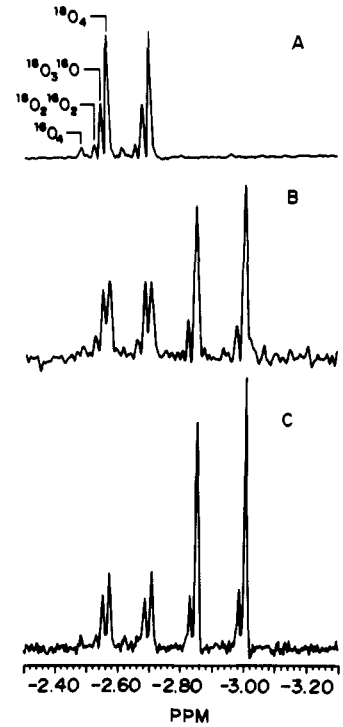
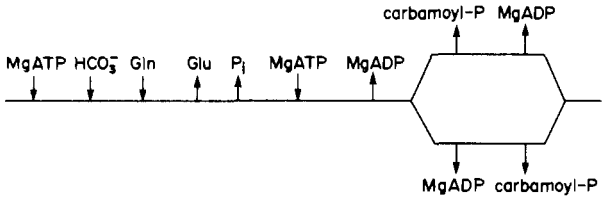


FIGURE 1: ^{31}P NMR spectra at 145.80 MHz of [γ-^{18}O]ATP (and [β-^{18}O]ADP) before and after CPS II catalyzed positional isotope exchange as outlined in Table III. The chemical shifts are relative to an external sample of 85% H_3PO_4/15% D_2O measured in a separate tube. (A) Experiment 1: [γ-^{18}O]ATP after incubation at 37 °C under reaction conditions in the absence of CPS. The number of atoms of ^{18}O in each of the peaks of a doublet of the γ-phosphorus group is noted. (B) Experiment 2: CPS II catalyzed positional isotope exchange of [γ-^{18}O]ATP in the presence of bicarbonate. A pair of doublets corresponding to the [β-^{18}O]ADP formed in the reaction is seen upfield from the γ-phosphorus resonances. (C) Experiment 3: CPS II catalyzed positional isotope exchange of [γ-^{18}O]ATP in the presence of bicarbonate and glutamine. Additional details are presented in Table III and in the text.

For the ATPase reaction, the ratio of micromoles of ATP exchanged to micromoles of ATP hydrolyzed (v_{ex}/v_{cat}) was 0.62.

At a high concentration of glutamine, ATP hydrolysis proceeded to 63% while the exchange was suppressed (experiment 3; Figure 1C). Thus, under conditions of the biosynthetic reaction, v_{ex}/v_{cat} decreased to 0.39. Since the addition of glutamine should result in a considerable increase in ATP hydrolysis, one might expect that a nearly saturating level of the amino acid would drastically lower v_{ex}/v_{cat}. However, as noted above, the ATPase activity of CPS II was 50% of the biosynthetic activity, and this ratio increased upon enzyme

Scheme I



storage. As such, some of the exchange observed in experiment 3 could have resulted from enzyme that was incapable of utilizing glutamine but could still catalyze ATP hydrolysis.

To investigate this possibility, PIX of [γ-^{18}O]ATP was measured in the presence of a high concentration of NH_4^+ in order to obviate the requirement of glutamine to effect the biosynthetic reaction. At 100 mM NH_4^+ (apparent K_m = 9 mM), the v_{ex}/v_{cat} was indeed diminished (0.16; experiment 4). In the biosynthetic reaction, the carbamoyl phosphate formed from [γ-^{18}O]ATP contains three atoms of ^{18}O, such that its reaction with the product [β-^{18}O]ADP by partial reversal of the net chemical reaction would contribute to the observed PIX in experiment 4. In experiment 5, in which aspartate and bacterial aspartate transcarbamoylase were included to rapidly remove [^{18}O]carbamoyl phosphate, v_{ex} was slightly less than in experiment 4, while no significant change in v_{ex}/v_{cat} was observed.

CPS II catalyzed PIX of [γ-^{18}O]ATP was investigated in the absence of bicarbonate. Since $K_{bicarbonate}$ is quite low in the ATPase reaction, care was taken to purge the samples of endogenous bicarbonate by degassing with N_2, and subsequently by the (continuous) enzymatic removal of HCO_3^- via the PEP carboxylase reaction. The removal of HCO_3^- was monitored spectrophotometrically to its completion by coupling the product oxaloacetate to the malate dehydrogenase reaction. Commercial preparations of PEP carboxylase were found to contain pyruvate kinase activity which presumably catalyzed extensive PIX of [γ-^{18}O]ATP (with pyruvate derived from PEP) in the absence of CPS II. Size exclusion chromatography removed most of this contaminating activity from PEP carboxylase, and in the absence of CPS II (experiment 6), PIX of [γ-^{18}O]ATP was very slight while no ADP could be detected in the ^{31}P NMR spectrum. The inclusion of 0.65 mg of CPS II into an otherwise identical reaction mixture (experiment 7) produced no ADP and very little additional exchange, demonstrating that CPS II did not significantly catalyze PIX of [γ-^{18}O]ATP in the absence of HCO_3^-.

Discussion

Kinetic Mechanism. The kinetic mechanism that is most consistent with the above data is the partially random ter bi uni ter quad quint mechanism shown in Scheme I. The binding of HCO_3^- is in rapid equilibrium with the E–MgATP complex. The mechanism is derived from the steady-state kinetic data by using the protocol of Cleland (1970), on the basis of the following observations:

(1) The intersecting initial velocity pattern of the ATPase reaction reflects a low-K_m MgATP (K_{MgATP_a} = 0.19 mM) which binds to the enzyme with a reversible connection to the binding of HCO_3^-. Absence of parabolic lines in this pattern implicates a single molecule of MgATP in the activation of HCO_3^-. In contrast, the parallel patterns observed for the biosynthetic reaction at high variable levels of MgATP demonstrate that no such reversible connection exists between the binding of a high-K_m MgATP (K_{MgATP_b} = 1.8 mM) and that of either HCO_3^- or glutamine. It is therefore likely that product release step(s) intervenes (intervene) between the binding of these substrates and the high-K_m MgATP. Since the concentrations of MgATP used in all studies of the biosynthetic reaction were (10–100)K_{MgATP_a}, the binding of $MgATP_a$ has been rendered kinetically "silent" at these concentrations, such that the kinetic patterns of the biosynthetic reaction reflect only the binding of $MgATP_b$.[3] These findings suggest that $MgATP_a$ is involved in the activation of HCO_3^- for its reaction with NH_3, while $MgATP_b$ contributes the phosphate group found in the product carbamoyl phosphate.

(2) Inhibition studies using AMP-PNP provide additional evidence that the kinetic behavior of $MgATP_a$ may be resolved from that of $MgATP_b$. The uncompetitive inhibition of AMP-PNP vs. both HCO_3^- and glutamine in the biosynthetic reaction indicates that these substrates bind to CPS II before the binding of $MgATP_b$, which binds to the same enzyme form as AMP-PNP. In the ATPase reaction, AMP-PNP, as its competitive inhibitor, binds to the same enzyme form as $MgATP_a$. This enzyme form is distinct from that to which $MgATP_b$ binds, since AMP-PNP vs. HCO_3^- in the ATPase reaction is noncompetitive. Thus, $MgATP_b$ binds after the binding of HCO_3^- and glutamine and the release of at least one product, while $MgATP_a$ binds before HCO_3^- to free enzyme.

(3) The conversion of the MgADP vs. HCO_3^- and MgADP vs. glutamine inhibition patterns from noncompetitive to uncompetitive by elevation of the fixed level of MgATP to saturation indicates that (a) at least one of the two molecules of MgADP binds to an enzyme form reversibly connected to those that bind HCO_3^- and glutamine and (b) that at least one molecule of MgATP (presumably $MgATP_a$) binds between the binding of this MgADP and these substrates. This indicates that both MgADP and $MgATP_a$ bind to free enzyme.

(4) The inhibition patterns of MgADP vs. MgATP in the ATPase and biosynthetic reactions are noncompetitive, in which K_{is} = 0.34 mM and 1.3 mM, respectively, while K_{ii} values are nearly identical (5 mM and 6 mM, respectively). This suggests that the intercept effects in the two patterns arise from the binding of one molecule of MgADP to a single enzyme–product complex that is downstream from the binding of $MgATP_b$. As such, one MgADP and $MgATP_a$ compete for free enzyme (K_{is} = 0.3 mM), while the second MgADP is released from a second set of central complexes that occurs subsequent to the binding of $MgATP_b$ (K_{ii} = 6 mM). In addition to its binding to free enzyme, the binding of the MgADP to this second site elicits the intercept effect in the MgADP vs. MgATP inhibition pattern of the ATPase reaction, which one would otherwise predict to be competitive.

(5) Two lines of evidence determine that the binding of HCO_3^- precedes that of glutamine: (a) MgADP vs. glutamine is noncompetitive at subsaturating HCO_3^- but is uncompetitive at saturating HCO_3^-; (b) the initial velocity pattern of HCO_3^- vs. glutamine is of the equilibrium ordered type, in which HCO_3^-, in rapid equilibrium with E–MgATP, exerts no concentration dependence on the initial rate at infinite glutamine (the lines of the pattern intersect on the $1/v$ axis) because all of the E–MgATP exists as E–MgATP–HCO_3^-–glutamine.

(6) Glutamate is a noncompetitive inhibitor vs. glutamine and HCO_3^- and is an uncompetitive inhibitor vs. $MgATP_b$.

[3] If MgATP were the first substrate to bind to enzyme in either the ATPase or biosynthetic reactions, the limiting value for K_m for either case would be V_{max}/k_1, in which the difference in V_{max} is approximately twofold. Assuming that k_1, the bimolecular rate constant for the addition of MgATP to E, is identical for both reactions, the K_m value for this first molecule of MgATP in the biosynthetic reaction would be approximately 0.38 mM, which is considerably lower than the concentrations of MgATP used in kinetic studies of the biosynthetic reaction.

Therefore, glutamate is the first product released from the first set of central complexes, and the release of a second product prior to the binding of $MgATP_b$ renders the inhibition of Glu vs. $MgATP_b$ uncompetitive.

(7) Phosphate is a noncompetitive inhibitor vs. $MgATP_b$ and an uncompetitive inhibitor vs. glutamine, and so phosphate is the second product released from the first set of central complexes. Phosphate and $MgATP_b$ bind to the same enzyme form, and as such, a competitive inhibition pattern is expected. However, in this pattern the intercept effect is quite small (K_{ii} = 170 mM) and may result solely from the weak binding of P_i to a phosphate-specific cleft in the binding site for MgADP, carbamoyl phosphate, or some other nonspecific site.

(8) The uncompetitive inhibition pattern of carbamoyl phosphate vs. $MgATP_b$ indicates that another product (MgADP) is released from enzyme before carbamoyl phosphate. However, the noncompetitive inhibition patterns of carbamoyl phosphate vs. both HCO_3^- and glutamine suggest that carbamoyl phosphate, as does the other MgADP, binds to free enzyme. The release of carbamoyl phosphate and the MgADP presumably derived from $MgATP_a$ from their respective enzyme–product complexes is random.

(9) Carbamoyl phosphate vs. $MgATP_a$ (ATPase reaction) is competitive, which confirms that both the substrate and product bind to free enzyme.

For the proposed kinetic mechanism, the lower branch of the random segment depicts the consecutive release of two molecules of MgADP. In such an event, one would expect to observe a parabolic effect upon the slopes and intercepts of lines from inhibition patterns in which MgADP was used as a product inhibitor. However, in all of the product inhibition patterns using MgADP, replots of the slopes and intercepts appeared to be linear. This lack of parabolism could arise from several possibilities: (a) the binding constant of one of the molecules of MgADP may be considerably higher than that of the other when they are bound consecutively; (b) the upper branch of the random pathway is the highly preferred one; or (c) it is the exclusive pathway, and the binding of carbamoyl phosphate to free enzyme constitutes the formation of an E–carbamoyl-P dead-end complex.

From the proposed kinetic mechanism, bicarbonate is activated and aminated, followed by the release of products, before the second molecule of MgATP is bound to the enzyme. These findings preclude a reaction mechanism in which glutamine-derived ammonia reacts directly with carbonyl bis(phosphate), the product of the reaction of HCO_3^- with 2 mol of MgATP. Thus, the CPS II catalyzed biosynthesis of carbamoyl phosphate may be envisioned as the overall result of three partial reactions: a "glutaminase" reaction (eq 11), a "carbamate synthetase" reaction (eq 12), and a "carbamate kinase" reaction (eq 13). In addition to carbamate, carboxyphosphate could be postulated as an enzyme-bound intermediate for this sequence of reactions.

$$\text{glutamine} + H_2O \rightarrow \text{glutamate} + NH_3 \quad (11)$$

$$MgATP + HCO_3^- + NH_3 \rightarrow MgADP + P_i + NH_2CO_2^- \quad (12)$$

$$MgATP + NH_2CO_2^- \rightarrow MgADP + \text{carbamoyl-P} \quad (13)$$

As originally proposed for carbamoyl-phosphate synthetase by Anderson and Meister (1966), the partial reactions of eq 11–13 were shown to be operative for the CPS from *E. coli* (Raushel et al., 1978). As elucidated, the kinetic mechanism of the bacterial CPS is nearly identical with that of CPS II, in which the release of products from the former conforms exclusively to the upper branch of the random segment of the latter. While glutamine binds in a random fashion to the smaller subunit of the bacterial enzyme, the ammonia derived from its hydrolysis intervenes in the reaction in an ordered manner at the same point at which glutamine binds to CPS II. For both enzymes, P_i is released prior to the binding of the second MgATP, implicating carbamate as one likely enzyme-bound reaction intermediate common to both reaction mechanisms. It is interesting that while bacterial CPS and CPS II are structurally quite disparate, the only significant differences in their respective kinetic mechanisms are in the binding of glutamine, the rapid-equilibrium binding of bicarbonate to CPS II, and the probable random release of products from CPS II. Inasmuch as V_{max}/E_t for CPS II ($\sim$0.5 s^{-1}) is approximately 16% of that of the *E. coli* CPS, it is possible that catalytic steps are more rate limiting for CPS II than for its bacterial counterpart and that this lower commitment to catalysis is reflected in the rapid-equilibrium binding of bicarbonate and the random release of some of its products.

While the glutamine-utilizing carbamoyl-phosphate synthetases appear to share common mechanistic features despite considerable structural dissimilarities, the ammonia-dependent CPS I from bovine liver operates by a very different kinetic mechanism (Elliott & Tipton, 1974a,b). Its mechanism is a strictly sequential, ordered one in which bicarbonate, ammonia, and both molecules of MgATP are bound to the enzyme prior to the release of products in the order: carbamoyl phosphate, MgADP, MgADP, and P_i. The results of isotope-trapping experiments suggested that for the mitochondrial enzyme (frog liver) the enzyme-bound HCO_3^- is activated by *both* molecules of MgATP prior to reaction with the bound NH_3, to yield a putative reaction intermediate similar in structure to carbonyl bis(phosphate) (Rubio & Grisolia, 1977).

Chemical Intermediates of the CPS II Catalyzed Reactions. The positional isotope exchange technique has been employed to elucidate the reaction intermediates of a number of ATP-utilizing enzymes (Rose, 1979), including the carbamoyl-phosphate synthetases of *E. coli* (Raushel & Villafranca, 1980) and rat liver mitochondria (Rubio et al., 1981). The exchange of an atom of ^{18}O from the $\beta\gamma$-bridge position of [γ-$^{18}O_4$]ATP to the β-nonbridge position attends the reversible formation of [β-^{18}O]ADP and a phosphorylated intermediate on the enzyme surface. The nucleophile that is the acceptor group of the phosphoryl transfer may be a substrate, an enzymatic residue, or both. The exchange results from the reversibility of the phosphoryl transfer and free rotation about the torsionally symmetric β-phosphate group of [β-^{18}O]ADP.

The maximum positional isotope exchange for CPS II was observed under conditions of the ATPase reaction. At 5 mM HCO_3^- the rate of exchange was 62% of that of the rate of ATP hydrolysis. Since, in the absence of bicarbonate, CPS II catalyzed positional oxygen exchange of [γ-^{18}O]ATP was found to be negligibly small, it would appear that bicarbonate is necessary to promote the exchange. The simplest interpretation of these results is that the identity of the nucleophilic group is bicarbonate, not an enzymatic residue.[4] This would suggest that the enzyme-bound reaction intermediate resulting from phosphoryl transfer is carboxyphosphate (Figure 2, pathway a). This species could serve as an intermediate for both the ATPase and biosynthetic reactions. The rate of formation or breakdown of carboxyphosphate is at least 62%

[4] Strictly, it is possible that bicarbonate must be bound to the enzyme in order to induce the phosphorylation of an enzymatic residue prior to covalent attack by bicarbonate.

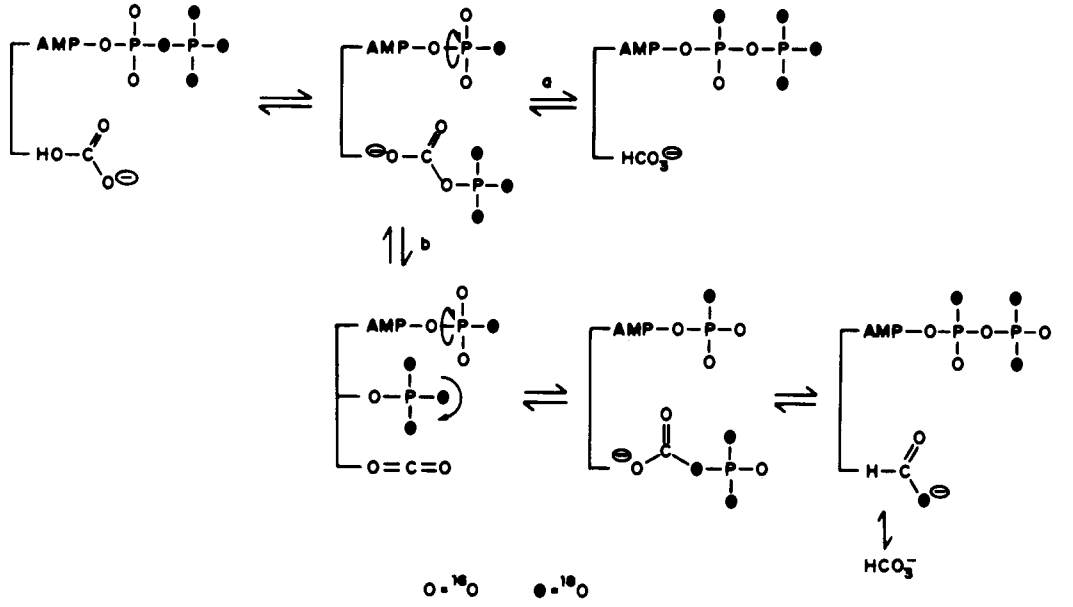


FIGURE 2: Mechanism for the CPS II catalyzed positional isotope exchange of [γ-^{18}O]ATP in the ATPase reaction. PIX resulting solely from the reversal of the formation of an E–MgADP–carboxyphosphate is depicted in pathway a. In pathway b, as a result of the intermediate formation of enzyme-bound CO_2 and rotationally unhindered P_i, three of four atoms of ^{18}O are retained in the re-formed bicarbonate, resulting in "washout" of the label in the re-formed [γ-^{18}O]ATP.

the rate of the forward ATPase reaction, while it greatly exceeds the immeasurably low rate of the reverse reaction. As such, carboxyphosphate is a kinetically competent intermediate of the ATPase reaction pathway; that is, its formation and breakdown are at least as rapid as the enzymatic reaction in the slower direction (Rose, 1979). By use of positional isotope exchange and rapid-quench techniques, carboxyphosphate was shown to be an intermediate of the ATPase reaction of the bacterial CPS, and its rate of formation on the biosynthetic reaction pathway was shown to be at least partly rate limiting (Raushel & Villafranca, 1979, 1980).

Once formed, it is possible that carboxyphosphate collapses to yield CO_2 and P_i as the major enzyme-bound reaction intermediates, as has been suggested by Sauers et al. (1975). In such an event, the resulting phosphate would contain a single atom of ^{16}O derived from bicarbonate. Raushel and Villafranca (1979) have pointed out that the continuous re-synthesis of ATP from an E–MgADP–CO_2–P_i complex would ultimately result in the "washout" of ^{18}O in the γ-phosphate group since 75% of the molecules of the re-formed [γ-^{18}O]ATP would contain this atom of ^{16}O obtained from an "unlimited" pool of [^{16}O]HCO_3^- (Figure 2, pathway b). The incorporation of this atom of ^{16}O into the [γ-^{18}O]ATP would be evident in its ^{31}P NMR spectrum to the extent that a sample that had reached only 20% of positional exchange equilibrium would evince a distribution pattern of ^{16}O that is quite distinct from a sample that did not suffer ^{18}O "washout". A quantitative analysis[5] of the γ-phosphorus signals in the spectrum of Figure 1B revealed that the distribution pattern of ^{16}O is more consistent with a sample that had not undergone "washout" of ^{18}O. Therefore, if an E–MgADP–CO_2–P_i complex exists on the ATPase reaction pathway, either the rotation of the bound P_i is greatly hindered or the formation of this complex is irreversible and all observed exchange results from the E–MgADP–carboxyphosphate complex.

Positional oxygen exchange is only slightly suppressed in the presence of a high concentration of glutamine. A diminution of exchange is expected since the rate of carbamoyl phosphate synthesis is twice that of the ATPase reaction. Moreover, given the ordered binding of substrates as depicted in Scheme I, one would expect that at such a high level of glutamine no reversal of a quaternary E–MgADP–$CO_2OPO_3^{3-}$–Gln complex could occur and exchange would be abolished. As noted above, upon extended storage CPS II displayed a decrease in V_{max}(biosynthetic)/V_{max}(ATPase), suggesting that its ability to hydrolyze glutamine has become impaired. To determine if the observed exchange resulted from "glutamine-incompetent" enzyme still capable of catalyzing the ATPase reaction, NH_4^+ was substituted for glutamine. At a concentration of NH_4^+ that was 10 times its Michaelis constant, the v_{ex}/v_{cat} ratio was 41% of that observed in the presence of 1 mM glutamine and 26% of that observed in its absence. As such, "glutamine-incompetent" enzyme may account for some, but not all, of the exchange observed with added glutamine.

The residual positional oxygen exchange observed at high concentrations of either glutamine or NH_4^+ may result from one of the following: (a) the [^{18}O]carbamoyl phosphate formed in the reaction accumulates and undergoes a CPS II catalyzed partial reverse reaction with [β-^{18}O]ADP; (b) the enzyme-bound reaction of the second molecule of [γ-$^{18}O_4$]ATP and carbamate is itself reversible and promotes exchange (Figure 3, pathway c); or (c) phosphoryl transfer in the E–MgADP–$CO_2OPO_3^{3-}$–Gln complex is easily reversed to afford E–MgATP–HCO_3^-–Gln. Of the first of these possibilities, no significant change in the ammonia-dependent positional isotope exchange was observed upon the addition of aspartate and aspartate transcarbamoylase in sufficient quantities to rapidly remove [^{18}O]carbamoyl phosphate, thereby indicating that a partial reverse reaction of carbamoyl phosphate and MgADP does not contribute to the exchange. In a separate experiment, MgADP and [$^{18}O_4$]carbamoyl phosphate (von der Saal et al., 1985) were incubated with CPS II to determine the extent of catalysis of this partial reverse reaction, which has been demonstrated with CPS from *E. coli* (Raushel & Villafranca, 1980). However, no exchange could be observed after 30 min, due partly to the chemical instability of the carbamoyl phosphate at 37 °C (DeBrosse and Meek, unpub-

[5] The analysis of the labeling patterns in exchanged [γ-^{18}O]ATP for several of the PIX experiments is available as supplementary material (see paragraph at end of paper regarding supplementary material).

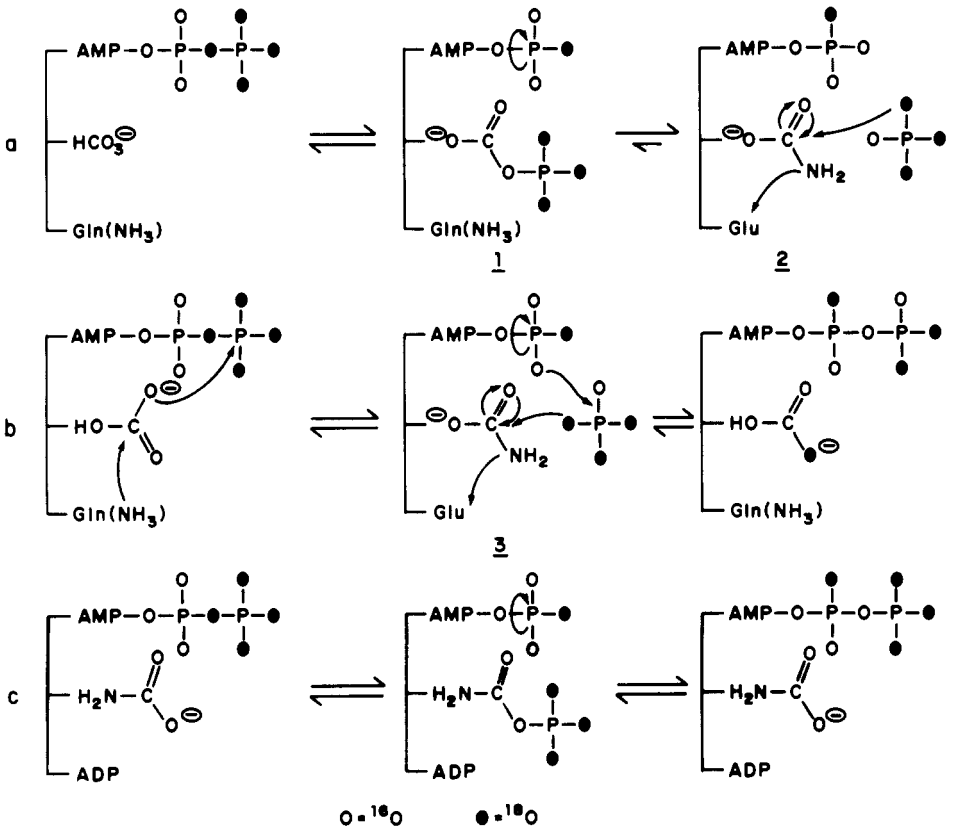


FIGURE 3: (Pathway a) Positional isotope exchange resulting from the stepwise formation of enzyme-bound carbamate. The resynthesis of [γ-^{18}O]ATP solely from complex 1 containing carboxyphosphate results in normal exchange patterns, while a reversal from complex 2 would contribute some "^{18}O washout" in the ATP. (Pathway b) Concerted mechanism of carbamate formation. The resynthesis of [γ-^{18}O]ATP from complex 3 will always result in "washout". (Pathway c) Positional isotope exchange of the second molecule of [γ-^{18}O]ATP in the biosynthetic reaction in which the phosphoryl acceptor is carbamate.

lished results). As such, we propose that the residual exchange results from one or both of the latter two possibilities.

For the biosynthetic reaction, the formation of carbamate from MgATP, HCO_3^-, and glutamine may involve the intermediate formation of carboxyphosphate or proceed by a concerted mechanism as has been proposed to account for the lack of MgATP:MgADP isotopic exchange when the ammonia source is absent (Guthohrlein & Knappe, 1969). For the stepwise mechanism (Figure 3, pathway a) positional isotope exchange could result from the reversal of complex 1 in which ammonia attacks the preformed carboxyphosphate or from complex 2 in which exchange would be subsequent to carbamate formation. The formation of complex 2 has been shown to be essentially irreversible for the bacterial CPS (Raushel & Villafranca, 1979). Due to the presence of free phosphate in complex 2, one may distinguish exchange patterns involving significant contributions from complex 2 from those resulting solely from complex 1 by the observation of "washout" of the ^{18}O label in the re-formed [γ-^{18}O]ATP as described above. For the concerted mechanism (Figure 3, pathway b) one would expect to observe substantial "washout" since all reaction reversal must involve [$^{18}O_3$,^{16}O]P_i (complex 3). While positional isotope exchange in the presence of glutamine (or NH_4^+) has apparently proceeded to a significant extent, the distribution of ^{16}O in the γ-phosphorus signals is in concert with an exchange mechanism that does not suffer scrambling of the label from P_i, as would attend exchange from the reversal of either complex 2 or complex 3 in Figure 3. From this, the positional isotope exchange observed for CPS II in the presence of glutamine or NH_4^+ most likely involves the reversal of a complex containing carboxyphosphate, perhaps containing some contribution of exchange from carbamate and the second molecule of [γ-^{18}O]ATP as mentioned above.

Since CPS II catalyzes the reverse biosynthetic reaction at a rate that is 1% of the forward reaction, the v_{ex}/v_{cat} values measured for PIX in the presence of either glutamine or NH_4^+ reflect that carboxyphosphate qualifies as a kinetically competent intermediate of the biosynthetic reaction pathway. These results, taken with the proposed kinetic mechanism, strongly support a stepwise reaction mechanism for CPS II (as outlined in eq 11–13), in which carboxyphosphate and carbamate are the likely enzyme-bound intermediates.

In summary, we have shown that despite considerable structural differences to its bacterial counterpart, the carbamoyl-phosphate synthetase II from the mammalian trifunctional CAD protein operates by a very similar kinetic mechanism, apparently employing the same enzyme-bound reaction intermediates. The few differences in the respective mechanisms could be accounted for by the lower rate of catalysis of the CPS II and its lack of a separate and independent subunit for the binding and hydrolysis of glutamine. This kinetic mechanism stands in contrast to that of the ammonia-dependent CPS I of mammalian liver mitochondria, suggesting that the transference of NH_3 from glutamine may militate the similar stepwise mechanisms of CPS II and the bacterial CPS.

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Supplementary Material Available

Five figures showing double-reciprocal plots of $1/v$ vs. $1/[MgATP]$ at 0.133 M HCO_3^- and 3.3 mM glutamine, $1/v$ vs. $1/[MgATP]$ at 10 mM HCO_3^- (no glutamine), HCO_3^- vs. MgATP at 3.3 mM glutamine, HCO_3^- vs. MgATP (no glutamine), and HCO_3^- vs. glutamine at 2 mM MgATP and a table showing mechanism-dependent labeling patterns of exchanged [γ-^{18}O]ATP (6 pages). Ordering information is given on any current masthead page.

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