Mechanism of Activation of Bicarbonate Ion by Mitochondrial Carbamoyl-Phosphate Synthetase: Formation of Enzyme-Bound Adenosine Diphosphate from the Adenosine Triphosphate That Yields Inorganic Phosphate†

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ABSTRACT: The mechanism of the reaction catalyzed by rat liver mitochondrial carbamoyl-phosphate synthetase has been studied by using [β-$^{18}O_2$]ATP and $HC^{18}O_3^-$, monitoring the isotopic composition of adenosine triphosphate (ATP) and inorganic phosphate (P_i) by high-resolution ^{31}P NMR spectroscopy. In the presence of both HCO_3^- and acetylglutamate, the enzyme catalyzes the exchange of oxygen atoms between the β,γ bridging and the β nonbridging positions of ATP. Addition of NH_3 stops the exchange. P_i released by the ATPase activity of the enzyme in the absence of NH_3 contains one oxygen atom from $HC^{18}O_3^-$ but there is no incorporation of ^{18}O into ATP. There is no significant incorporation of [^{14}C]ADP or $^{32}P_i$ into ATP. It is concluded that in the enzyme·ATP_A·HCO_3^-·ATP_B complex formed in the presence of ATP and HCO_3^- there is reversible transfer of the γ-PO_3 group of ATP_A (the molecule that yields P_i) to HCO_3^- without dissociation of products. The β-PO_3 of the enzyme-bound ADP that is formed can rotate. Virtually all of the complex appears to be in the form in which ATP_A is cleaved, but in the absence of NH_3, ATP is reconstituted and dissociates from the complex on at least 75% of the occasions. On the remainder, the carbonyl phosphate is cleaved in an irreversible process that yields P_i and a low-energy form of carbonic acid (probably HCO_3^-). NH_3 reacts rapidly and irreversibly with the complex, and at saturation the rate (>10 times the rate of P_i release in the absence of NH_3) is sufficient to prevent dissociation of ATP_A. In the absence of HCO_3^- an enzyme·ATP_A·ATP_B complex is formed, but cleavage of the bond between β,γ bridging oxygen and P_γ of ATP_A does not occur.

Mitochondrial carbamoyl-phosphate synthetase catalyzes the reaction $2ATP + HCO_3^- + NH_3 \rightarrow 2\ ADP + P_i + O_3POCONH_2$. In the absence of NH_3 it exhibits HCO_3^--dependent ATPase activity, which was interpreted as evidence for the formation of "active CO_2" as a first step (Metzenberg et al., 1958).

Our work with the rat liver enzyme (Rubio et al., 1979; Britton et al., 1979) which extended earlier work with the frog liver enzyme (Rubio & Grisolia, 1977) has shown that in the presence of ATP and HCO_3^- an enzyme·ATP_A·HCO_3^-·ATP_B[1] complex is formed that has the following properties: it reacts rapidly with NH_3 to give carbamoyl phosphate and P_i; it reacts slowly with H_2O to release P_i from ATP_A; and when it is treated with $HClO_4$, ATP_A decomposes into ADP and P_i. These properties were taken as evidence for a bond between HCO_3^- and the terminal phosphoryl group of ATP_A as proposed by Jones & Spector (1960) [see also Jones (1976)]. Formation of such a bond may be associated with breakage of the bond between the P_γ and the β,γ bridging oxygen of ATP so that ADP is formed at the active center. It is probable that if ADP is formed it will not dissociate reversibly from the active center since ADP–ATP exchange is not observed with the frog liver enzyme (Marshall et al., 1958). However, we have shown that ATP_A exchanges rapidly with ATP in solution ($t_{1/2} \ll 10$ s). Thus it should be possible to test whether ADP is formed at the ATP_A binding site by the positional isotope exchange technique of Midelfort & Rose (1976). This paper, therefore, reports observations on the migration of ^{18}O from [β-$^{18}O_2$]ATP into the β,γ bridging position of ATP using ^{31}P NMR spectroscopy. Data on the transfer of ^{18}O to ATP and P_i from $HC^{18}O_3$ and on [^{14}C]ADP–ATP and $^{32}P_i$–ATP exchanges by the enzyme are also reported.

A positional exchange study with *E. coli* carbamoyl-phosphate synthetase has already been published (Wimmer et al., 1979). The present study seemed particularly desirable since there are many differences between *E. coli* and the mitochondrial synthetase, and it is not clear whether they share a basic mechanism or are even homologous enzymes.

Materials and Methods

Rat liver carbamoyl-phosphate synthetase I isolated as described (Rubio et al., 1979) contained a trace of myokinase and some impurities capable of catalyzing ATP–ADP exchange. They were removed by passage of the enzyme (1 mL) through a Sephadex G-200 column (0.9 × 60 cm) equilibrated and eluted with a solution containing 50 mM Tris-HCl, pH 7.2, 1 mM dithioervthritol, 20% (v/v) glycerol, and either 50 mM KCl or 50 mM NaCl. The enzyme was essentially free of myokinase, but Ap_5A[2] (2 mM) was included in most incubations. At this concentration it did not inhibit appreciably carbamoyl-phosphate synthesis or ATP hydrolysis by the enzyme. ADP was also routinely added to quantitate ADP-ATP exchange and to reduce by isotope dilution any positional

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[1] ATP_A yields P_i and ATP_B provides the phosphoryl group of the carbamoyl phosphate.

[2] Abbreviations used: Ap_5A, P^1,P^5-di(adenosine 5′-)pentaphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; AG, *N*-acetyl-L-glutamate. Enzymes used: carbamoyl-phosphate synthetase (ammonia), EC 6.3.4.16; carbamoyl-phosphate synthetase (glutamine-hydrolyzing), EC 6.3.5.5; ornithine carbamoyltransferase, EC 2.1.3.3.

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isotope exchange due to the ADP–ATP exchange.

ATP was labeled with ^{18}O in the nonbridging oxygens at P_β ([β-$^{18}O_2$]ATP) (Lowe & Sproat, 1981). NMR spectroscopy indicated the following species to be present: [β-$^{18}O_2$]-ATP, 50.7%; [β-^{18}O]ATP, 41.0%; and [β-$^{16}O_2$]ATP, 8.3%. $HC^{18}O_3^-$ was prepared by dissolving $KHCO_3$ (75 μmol) in 50 μL of $H_2{}^{18}O$ and allowing the solution to stand overnight at room temperature. The water, which was shown by mass spectrometry to contain 67.6 atom % ^{18}O after equilibration with $KHCO_3$ (W. G. Gunn, Chemistry Department, Queen Elizabeth College, London), was removed in a vacuum desiccator over P_2O_5 shortly before use.

[8-^{14}C]ADP (55 mCi/mmol) and $^{32}P_i$ were obtained from the Radiochemical Centre, Amersham, England.

Ornithine carbamoyltransferase was from Sigma. PEI–cellulose (20 × 20 cm) thin-layer plates were from Merck. Other chemicals were from Sigma, Boehringer, and BDH.

Unless specified, all assays were as described previously (Britton et al., 1979).

Incubations of the substrates with the enzyme were generally carried out at 37 °C in capped plastic syringes (2.5 mL) with the plunger adjusted to minimize the air space. Alternatively, plastic microcentrifuge tubes (1.5 mL) were used (Eppendorf, Germany) filled to the level of the plastic stopper.

Samples (50–100 μL) taken at the start and end of the incubations were precipitated with perchloric acid at 0 °C and P_i, ATP, and ADP determined. The reaction in the remainder of the mixture was stopped with 0.75 volume (25% excess over Mg^{2+}) of ice-cold 50 mM Na_2EDTA adjusted to pH 7.6 with triethylamine. The protein was then denatured by vigorous agitation with 4 mL of $CHCl_3$ for 5 min (Lowe & Sproat, 1978). The supernatant was removed after centrifugation and the chloroform layer washed with triethylammonium bicarbonate (3 × 5 mL, 100 mM pH 7.6). The washings were combined with the supernatant and applied to an anion-exchange column (DEAE-Sephadex A-25 HCO_3^- form, 0.8 × 13 cm) equilibrated with 0.2 M triethylammonium bicarbonate, pH 7.6. The column was eluted at room temperature with a linear gradient of triethylammonium bicarbonate (0.2–0.6 M, 500 mL, pH 7.6) at a flow rate of 21 mL/h. AMP, ADP, and ATP were identified by UV absorption and their positions on the chromatogram. Samples from each peak were taken for radioactivity measurement. ATP was taken to dryness in a film evaporator with addition of methanol (3 × 5 mL) to remove triethylammonium bicarbonate. The same procedure was used to isolate P_i. The peak of P_i was located by the addition of tracer quantities of $^{32}P_i$.

ATP samples for ^{31}P NMR spectroscopy were dissolved in aqueous 2-amino-2-methylpropane-1,3-diol hydrochloride buffer (2 mL, 100 mM, 25% D_2O, pH 9.0) containing EDTA (10 mM) and were placed in 10-mm external diameter precision NMR tubes (Wilmad Glass Co., Inc., Buena, NJ) fitted with Teflon vortex suppressors.

^{31}P NMR spectra were recorded at 121.493 MHz on a Bruker WH-300 WB Fourier transform spectrometer, using quadrature detection. Signal averaging was performed by an Aspect 2000 computer interfaced with the spectrometer. Field-frequency locking was provided by the deuterium resonance of D_2O. ^{31}P NMR parameters were 2000–6000 transients, bandwidth 3012 Hz, memory size either 8K or 16K, pulse repetition rate 1.36 or 2.72 s^{-1}, pulse angle 69 °, and broad-band proton noise decoupling. Spectra were resolution enhanced by Gaussian multiplication of the free induction decay using line broadening between −0.8 and −1.4 Hz and Gaussian broadening between 0.15 and 0.3 depending on the signal/noise ratio. The Gaussian multiplied free induction decay was then zero filled to 32K memory size and Fourier transformed.

ADP–ATP exchange was measured by adding a tracer quantity of [^{14}C]ADP to the reaction mixtures and determining the relative amounts of radioactivity in the ATP and ADP peaks after ion-exchange chromatography as described above. Alternatively, the reaction was stopped with $HClO_4$, the mixture neutralized with Tris base, and an aliquot applied to a thin-layer PEI–cellulose sheet. After removal of salts with methanol, the chromatogram was developed with ammonium formate (0.6 M, pH 3.5), the nucleotides were identified by UV fluorescence, and the appropriate areas were removed by scraping.

P_i–ATP exchange was measured by incubation with $^{32}P_i$ followed by selective precipitation of P_i and counting of the supernatant. Samples (100 μL) from the reaction mixture were added to 200 μL of ice-cold 0.5 M $HClO_4$. All subsequent steps were at 0 °C and care was taken to ensure thorough mixing. After centrifugation, 100 μL of the supernatant was added to a freshly prepared mixture of 1 mL of 0.15 M $HClO_4$, 0.4 mL of 20 mM ammonium molybdate, and 0.1 mL of 100 mM triethylamine hydrochloride (pH 5). KH_2PO_4 (100 μL, 10 mM) was then added, and after 30 min the mixture was centrifuged. The supernatant (1.5 mL) was transferred to a second tube and 50 μL of KH_2PO_4 (10 mM) added. After 1 h, the mixture was centrifuged, and 1 mL of the supernatant and 0.2 mL of 2 N KOH were transferred to a scintillation vial for counting. All values were corrected for the blank (~0.01% of the radioactivity in P_i) obtained by taking a sample from the incubation mixture at the start of the incubation.

Results

When rat liver carbamoyl-phosphate synthetase is incubated in the presence of acetylglutamate with bicarbonate and [β-$^{18}O_2$]ATP (i.e., ATP labeled with ^{18}O in the nonbridging positions at P_β), the ^{31}P NMR spectrum shows a new doublet just upfield of P_γ corresponding to the isotope shift expected for bonding of P_γ to ^{18}O in the β,γ bridging oxygen (Figure 1). Thus, the bond between the β,γ bridging oxygen and the P_γ must be broken in the enzyme–ATP complex and the β-PO_3 of the ADP that is formed must rotate before resynthesis of ATP (Figure 2).

Quantitation of the spectral changes show that the rate of positional isotopic exchange is proportional to the amount of enzyme (Table I). Omission of bicarbonate completely abolishes exchange and the hydrolysis of ATP. Changing the concentration of K^+ from 110 mM (experiments I–III) to 5 mM (experiments IV–VI) has little effect on the rates of ATP hydrolysis or of the positional exchange. However, at 5 mM K^+, both activities are almost entirely dependent upon the presence of acetylglutamate. This excludes the possibility that these findings are due to contamination by other ATPases. At 110 mM K^+, omission of acetylglutamate reduces both activities of the enzyme by less than 50%. The activity that remains is due to activation by the high K^+ concentration and the glycerol present in the enzyme preparation (H. G. Britton, V. Rubio, and S. Grisolia, unpublished results). The addition of NH_3 abolishes positional isotopic exchange in the presence of ornithine transcarbamoylase with and without ornithine. This result is to be expected if NH_3 reacts rapidly with an intermediate formed by the enzyme from HCO_3^- and ATP_A in an irreversible process. In these last experiments, the rate of P_i production by the enzyme was increased 2.2–3.2 times by the presence of 35 mM $(NH_4)_2SO_4$.

Table I: Exchange of β,γ Bridging–β Nonbridging Oxygen of ATP Catalyzed by Rat Liver Carbamoyl-Phosphate Synthetase[a]

expt	conditions	initial ATP (μmol)	hydrolysis of ATP, V_H (nmol/min)	positional exchange[c] (% equilibrium)[b]	exchange of ATP, V_{EX} (nmol/min)	V_{EX} per enzyme unit (μmol·min^{-1}·unit^{-1})	$\frac{V_{EX}}{V_H + V_{EX}}$
I	complete, 120 min	19.8	80	83.4	217	2.7	0.73
II	complete, 64 min	12.6	53	61.7	162	3.1	0.75
	–AG, 120 min	12.6	31	60.2	81	1.5	0.72
	$-HCO_3^-$, 120 min	12.6	<2[d]	<4.2[e]	<4	<0.1	
III	$+NH_3$, 30 min	12.6	58[f]	<4.2[e]	<15	<0.9	<0.21
IV	complete, 120 min	13.5	31	63.0	96	3.1	0.76
	–AG, 120 min	13.5	2	13.5	16	0.5	~0.89[g]
V	complete, 60 min	17.9	68	57.7	226	3.3	0.77
	$-HCO_3^-$, 60 min	17.9	<4[d]	<4.2[e]	<13	<0.2	
VI	$+NH_3$, 45 min	16.8	50[f]	<4.2[e]	<14	<0.6	<0.22

[a] The enzyme was incubated at 37 °C with the indicated amount of [β-$^{18}O_2$]ATP in 2 mL (experiment I) or 1.5 mL (other experiments) of a solution of 7% (v/v) glycerol, 18 mM Tris-HCl, 1.4 mM dithioerythritol, 30 mM $MgCl_2$, 10 mM acetylglutamate, 50 mM $KHCO_3$ or $NaHCO_3$, 17 mM KCl or NaCl, 2 mM $LiAp_5A$, and 4.5 mM [^{14}C]ADP (1.07×10^6 counts/min), pH 7.2. When acetylglutamate or bicarbonate was omitted, equivalent amounts of KCl or NaCl were added. Enzyme units are micromoles of P_i released per minute by the ATPase activity of the enzyme in the complete incubation mixture. The amounts of enzyme used were (experiment I) 0.5 mg, (experiment II) 0.28 mg, (experiment III) 0.09 mg, (experiment IV) 0.16 mg, (experiment V) 0.35 mg, and (experiment VI) 0.07 mg. Experiments I–III, 110 mM K^+ and 25 mM Na^+. Experiments IV–VI, 130 mM Na^+ and 5 mM K^+. Experiment III, 35 mM $(NH_4)_2SO_4$, 13 mM ornithine, and 16 units/mL ornithine carbamoyltransferase. Experiment VI, 35 mM $(NH_4)_2SO_4$ and 16 units/mL ornithine carbamoyltransferase. Incubations were started by the addition of the enzyme and were carried on for the time specified. For further details see Materials and Methods. ATP consumption and P_i production were equal within experimental limits. Radioactivity in the ATP peak was (as % of the radioactivity of the [^{14}C]ADP added) the following: I complete, <0.1%; II complete, <0.1%; II –AG, 1.2%; II $-HCO_3^-$, 1.5%; III $+NH_3$, <0.1%; IV complete, 0.3%; IV –AG, 1.0%; V complete, 0.5%; V $-HCO_3^-$, <0.1%; VI $+NH_3$, <0.1%. [b] At equilibrium ^{18}O enrichment of the β,γ bridging oxygen was 47.5%. [c] Calculated according to $V_{EX} = X[ATP]_0 \ln(1-F)/[t \ln(1-X)]$, where X = fraction of ATP lost; $[ATP]_0$ = ATP concentration at time 0; F = positional exchange attained, as a fraction of the equilibrium value (Wimmer et al., 1979). [d] Assuming that the minimal amount of P_i (or of ADP) produced that can be detected is 0.25 μmol. [e] Assuming that ^{18}O in the β,γ bridging oxygen cannot be detected below 2% enrichment. [f] On the basis of 1 mol of ATP hydrolyzed per mol of carbamoyl phosphate synthesized. [g] Approximate value because of limited hydrolysis and exchange.

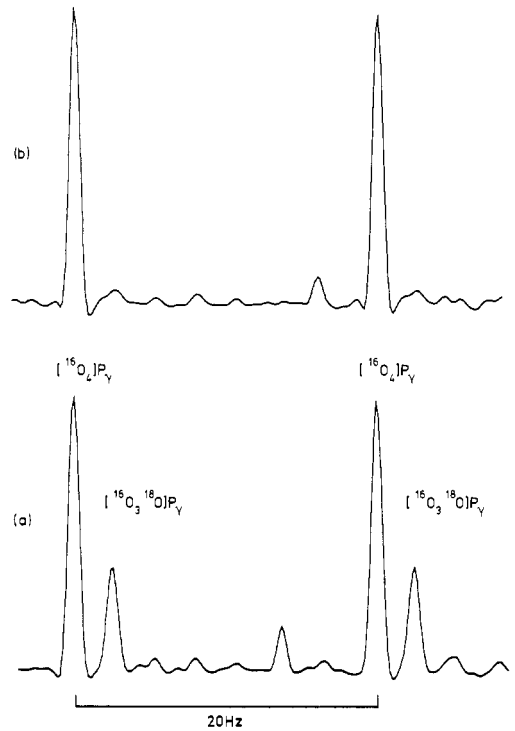

FIGURE 1: ^{31}P NMR spectra of P_γ of [$^{18}O_2$]ATP isolated from experiment V (Table I): (a) is the spectrum of [$^{18}O_2$]ATP after incubation with the complete system for 60 min; (b) is the spectrum [$^{18}O_2$]ATP isolated after incubation with the complete system minus bicarbonate for 60 min. The assignments are shown on the spectra. A small amount of ADP is formed during the isolation of the [$^{18}O_2$]ATP, and one limb of the P_β doublet appears in each spectrum.

If the ADP that is formed at the active center of the enzyme in the above experiments can dissociate reversibly, then ADP–ATP exchange should occur. [^{14}C]ADP was added to the incubations to test for this. Only very small amounts of label were found in ATP (<1.5% of the radioactivity in ADP; see legend to Table I) corresponding to an exchange rate of <15 nmol/min per ATPase unit. Such a rate would not have given any detectable positional exchange. Indeed, it was probably not due to the synthetase since it varied from preparation to preparation and was the same whether or not acetylglutamate, NH_3, and/or HCO_3^- were present. Further, in the final step of enzyme purification (see Materials and Methods), the ADP–ATP exchange activity appeared as a separate overlapping peak which followed the enzyme. The first fractions containing enzyme activity were without measurable ADP–ATP exchange activity.

Table II: $^{32}P_i$–ATP Exchange with Rat Liver Carbamoyl-Phosphate Synthetase[a]

	initial concentration		production of P_i (mM)	radioactivity incorporated into ATP (% of total radioactivity)
	ATP (mM)	ADP (mM)		
A	8.80	1.79	1.88	0.043
B	8.85	1.79	1.73	0.033

[a] $^{32}P_i$ (1 μCi) was incubated for 2 h at 37 °C with carbamoyl-phosphate synthetase in 0.335 mL of a buffer containing the indicated amounts of ATP and ADP, 15 mM Tris-HCl, 1.2 mM dithioerythritol, 4.6 mM acetylglutamate, 45 mM HCO_3^-, 30 mM $MgCl_2$, 1.9 mM $LiAp_5A$ and 7% (v/v) glycerol, pH 7.2. In (A) 4.8 mM K^+ and 111 mM Na^+. In (B) 81 mM K^+ and 24 mM Na^+. Initial P_i concentrations were 0.29 mM in (A) and 0.25 mM in (B). For details of assays see Materials and Methods.

With the formation of ADP at the active center, the possibility of a reversible dissociation of P_i should also be considered. Experiments were therefore carried out with $^{32}P_i$, and the incorporation of radioactivity into ATP was determined (Table II). The small amount of incorporation corresponded to a negligible amount of exchange (<0.3 nmol/μmol of P_i formed). The incorporation of label in similar experiments at pH 7.8 was also very small (Table III). These findings are consistent with the observations of Guthöhrlein & Knappe (1969).

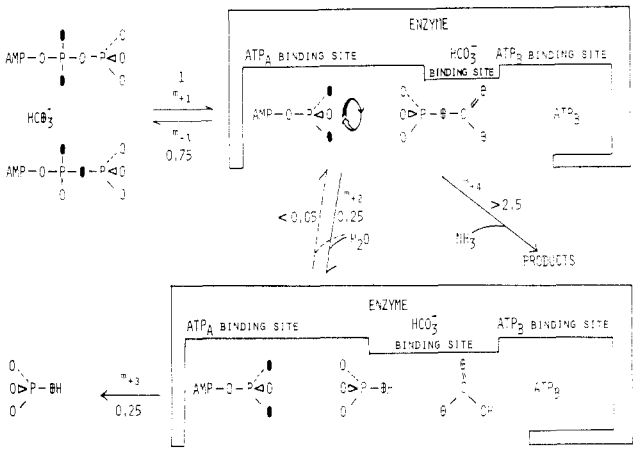

FIGURE 2: Diagram showing steps involved in positional isotope exchange and in ^{18}O incorporation from $HC^{18}O_3^-$ into P_i catalyzed by rat liver carbamoyl-phosphate synthetase in the presence of HCO_3^- and acetylglutamate. ● and ⊕ indicate ^{18}O. The numbers represent relative fluxes through the different pathways. Initially ATP is labeled with ^{18}O (●) exclusively in the nonbridging oxygens attached to the P_β. It combines with the enzyme at the ATP_A binding site (ATP_A yields P_i in the overall reaction). The rate of this reaction of ATP with the enzyme is arbitrarily set at unity. Since HCO_3^- and ATP_B (the ATP that yields carbamoyl phosphate in the overall reaction) are also bound, an enzyme·ATP_A·HCO_3^-·ATP_B complex is initially formed (not represented for simplicity). The γ-PO_3 of ATP_A is transferred to HCO_3^- to give the complex shown, which is the main species present. Rotation of β-PO_3 may now occur so that when the reactions are reversed, ^{18}O is present in the β,γ bridging positions as well as in the P_β nonbridging positions of the ATP. The complex may react with H_2O to give an enzyme·ADP_A·P_i·HCO_3^-·ATP_B complex from which P_i is released. With each turnover of the enzyme, equilibration of enzyme-bound HCO_3^- with HCO_3^- in solution must occur so that ^{18}O from $HC^{18}O_3^-$ is incorporated into P_i as observed. Rotation of P_i in the complex and reversal of the reaction could lead to incorporation of ^{18}O into ATP from $HC^{18}O_3^-$. Since this did not occur, an upper limit is given for the velocity of this reaction (see Appendix). Lack of incorporation of ^{18}O into ATP also indicates that a complex containing CO_2 as the activated form of bicarbonate (see text) is unlikely to be formed. The velocity of the reaction of the enzyme·ADP_A·O_3POCO_2·ATP_B complex with ammonia must be at least 10 times faster than the rate of formation of the enzyme·ATP_A·P_i·HCO_3^-·ATP_B complex to account for the absence of positional isotope exchange in the presence of 70 mM NH_4^+.

In the normal enzyme reaction of carbamoyl-phosphate synthesis, an oxygen atom in the P_i that is formed is derived from HCO_3^- (Jones & Spector, 1960). Data with $HC^{18}O_3^-$ show that this is also the case with the ATPase reaction (Table III). The experiments were carried out at pH 7.8 to minimize exchange of $HC^{18}O_3^-$ with the water. The ^{31}P NMR spectrum of the P_i indicated the incorporation of not more than one ^{18}O atom per molecule of P_i. In the absence of acetazolamide, the data are consistent with a $t_{1/2}$ for exchange of HCO_3^- with water of ~2 min if all P_i molecules contain one oxygen atom derived from HCO_3^-. This value for $t_{1/2}$ is shorter than that reported at this temperature and pH for the uncatalyzed reaction [7.8 min; C. K. Tu, quoted in Wimmer et al. (1979)]. However, in the presence of acetazolamide (an inhibitor of carbonic anhydrase), the ^{18}O content of the P_i (34.8%) was considerably increased and in agreement with a calculated value of 36.1% for the measured value of $t_{1/2}$. The smaller values for $t_{1/2}$ were presumably related to traces of carbonic anhydrase in the enzyme preparation. No labeling of ATP with ^{18}O was observed in any of the experiments (Table III).

Table III: Incorporation of ^{18}O into P_i and ATP from $HC^{18}O_3^-$ [a]

expt	acetazolamide (mM)	P_i produced (μmol)	$[^{18}O^{16}O_3]P_i$ (% of total P_i)	[γ-^{18}O]ATP (% of total ATP)
I	0	12.9	12.5[b]	<2
II	0	10.0	11.6[b]	<2
III	0.9	7.1	34.8[b]	<2

[a] In experiment I, 0.7 mL of a solution of 14.3% (v/v) glycerol containing the enzyme, 25 μmol Tris-HCl, 25 μmol KCl, 2 μmol dithioerythritol, and 15.5 μmol acetylglutamate, pH 7.2, was incubated at 37 °C for 16 min and then mixed with 0.96 mL containing 28 μmol of ATP, 50 μmol of $MgCl_2$, 50 μmol of Tris-HCl, and 1 μCi of $^{32}P_i$ (10 nmol). The mixture was rapidly added to 75 μmol of $KHC^{18}O_3^-$ (solid, 67.6% ^{18}O enrichment) and the incubation (final pH 7.8) continued for 16 min at 37 °C. The reaction was stopped by addition to ice-cold EDTA and ^{18}O in P_i and in ATP was assayed (see Materials and Methods). Samples were also taken at the start and at the end of the incubation to measure P_i and ADP release. In experiment II, 0.55 mL of a solution of 12.7% (v/v) glycerol containing the enzyme, 17.5 μmol of Tris-HCl, pH 7.2, 17.5 μmol of KCl, 1.85 μmol of dithioerythritol, and 15 μmol of acetylglutamate were used for the initial incubation. The remainder of the procedure and the conditions were as in experiment I. In experiments III, the initial incubation was carried out with 1.2 mL of a solution of 8.3% (v/v) glycerol containing the enzyme, 75 μmol of Tris-HCl, 25 μmol of KCl, 2 μmol of dithioerythritol, 15.5 μmol of acetylglutamate, and 1.5 μmol of acetazolamide, pH 7.8. After 16 min at 37 °C, 23 μmol of ATP, 50 μmol of KCl, 50 μmol of $MgCl_2$, and 1 μCi of $^{32}P_i$ (10 nmol) were added in 0.46 mL and the remainder of the procedure and conditions were as in experiment I. The radioactivity incorporated into ATP was <0.4% of that in the P_i in all experiments. [b] No $[^{18}O_2{}^{16}O]P_i$, $[^{18}O_3{}^{16}O]P_i$, or $[^{18}O_4]P_i$ species observed: limit of detection ~2% of total P_i.

Discussion

The finding of positional isotopic exchange catalyzed by rat liver carbamoyl-phosphate synthetase demonstrated reversible cleavage of the bond between the β,γ bridging oxygen and P_γ of ATP. Under the conditions of exchange, most of the enzyme was in the form of the enzyme·ATP_A·HCO_3^-·ATP_B complex[3] [$K_D(ATP_A)$ ~ 0.2 mM; $K_D(ATP_B)$ ~ 10 μM; $K_D(HCO_3^-)$ = 0.8 mM; Britton et al. (1979)]. The formation of the complex requires acetylglutamate, and the positional isotope exchange is also dependent upon this activator. ATP_B in the complex can only leave if HCO_3^- or ATP_A dissociates. Thus ATP_B exchanges very slowly with ATP in solution ($t_{1/2}$ > 50 s, 25 °C). This rate of exchange at 25 °C gives a value for V_{EX} (Table I) of <0.02 μmol/min per enzyme unit. Therefore, even allowing for the higher temperature in the present experiments (37 °C), ATP_B can only make a negligible contribution to the observed positional exchange. ATP_A in the complex exchanges rapidly ($t_{1/2} \ll 10$ s), and it must be concluded that all of the measured positional isotope exchange takes place at the ATP_A binding site. Since there was a negligible amount of ADP–ATP exchange, it must also be concluded that the ADP that is formed at the ATP_A binding site is unable to dissociate reversibly. Reversible dissociation of P_i from the active center when enzyme-bound ADP is formed can also be excluded since there is no $^{32}P_i$–ATP exchange catalyzed by the enzyme. Cleavage of ATP_A therefore is not followed by reversible dissociation of the products.

Lowe & Sproat (1978) observed positional isotope exchange in the absence of a phosphoryl acceptor with pyruvate kinase, suggesting that the terminal phosphate of ATP becomes a metaphosphate ion when bound to the enzyme. However, this was not observed with creatine kinase (Lowe & Sproat, 1980). In the present experiments there was no positional exchange or ATPase activity in the absence of HCO_3^-. Nevertheless, ATP_A and ATP_B still bind to the enzyme although ATP_A does

[3] Most of this complex will be in the form enzyme·ADP_A·O_3POCO_2·ATP_B (see below and Figure 2).

not hydrolyze when the complex is treated with $HClO_4$ [K_D-(ATP_A) = 0.2–0.6 mM, $t_{1/2}$ for exchange with ATP in solution ~1 s; $K_D(ATP_B)$ ~ 10 μM, $t_{1/2}$ for exchange with ATP in solution 5–10 s; Rubio et al. (1978); Britton et al. (1979)]. Cleavage of the bond between the β,γ bridging oxygen and P_γ of ATP appears to depend therefore upon enzyme-bound HCO_3^- acting as a phosphoryl acceptor, and this is supported by the finding of the incorporation of ^{18}O from $HC^{18}O_3$ into P_i in the ATPase reaction. It may, thus, be concluded that there is reversible formation of a complex containing carbonyl phosphate, ADP, and a molecule of ATP_B. This complex must be in equilibrium with a complex in which ATP_A is intact. However, in our previous work (Britton et al., 1979), identical "bursts" of $^{32}P_i$ were obtained when the enzyme after a pulse incubation with HCO_3^- and [γ-^{32}P]ATP was denatured with $HClO_4$ or reacted with NH_3. This suggests that the equilibrium is largely in favor of the carbonyl phosphate complex. It is remarkable that despite the formation of this complex the K_D for ATP_A is very similar to that found in the absence of HCO_3^-.

The ratio $V_{EX}/(V_{EX} + V_H)$ (Table I) gives an indication of the frequency of dissociation of ATP_A from the enzyme·ATP_A·HCO_3^-·ATP_B complex relative to the frequency of binding. It will be a minimum value if rotational of the β-PO_3 of enzyme-bound ADP is restricted. Thus, there is at least a 75% probability that a molecule of ATP which binds at the ATP_A binding site will dissociate again as ATP. Since the turnover of the enzyme as an ATPase is ~1 s^{-1} per enzyme dimer at 37 °C, the values of V_{EX} (Table I) indicate that ATP_A dissociates from the enzyme at a rate of ~3 s^{-1}. If both subunits are enzymatically active (Britton et al., 1979), the rate of dissociation of ATP_A from each complex is 1.5 s^{-1}. This figure is very similar to the extrapolated value (~2 s^{-1}, 37 °C) for the release of $H^{14}CO_3^-$ from the frog liver enzyme·ATP_A·$H^{14}CO_3^-$·ATP_B complex (Rubio & Grisolia, 1977). It suggests that the dissociation of ATP_A and HCO_3^- from the complex are both determined by a common rate-limiting step. This could be the reconstitution of the β,γ bond of the ATP.

In the presence of ammonium ions (70 mM, pH 7.2) positional isotope exchange is abolished [$V_{EX}/(V_{EX} + V_H) < 0.22$]. Thus if NH_3 does not alter the rate of dissociation of ATP_A, it must react reversibly with the complex at least 10 times more rapidly that H_2O (Figure 2). This corresponds to a pseudo-first-order rate constant for the reaction of the complex with NH_3 of >10 s^{-1}. A rapid rate of reaction of this order is demanded by our pulse–chase data. Further, Rubio & Grisolia (1977) measured the rate directly with the frog liver enzyme and obtained a value of 8×10^4 M^{-1} s^{-1} for the second-order rate constant at 25 °C. From this figure, a pseudo-first-order rate constant of 47 s^{-1} at 25 °C can be calculated for the present conditions.

The incorporation of ^{18}O into P_i in the ATPase reaction is quantitatively in agreement with HCO_3^- as the source of one of the oxygens in P_i, as previously found for the synthetase reaction (Jones & Spector, 1960). This requires that with each turnover of the enzyme HCO_3^- bound to the enzyme equilibrates with HCO_3^- in solution. The rate of exchange of HCO_3^- in the complex may not be sufficiently rapid if it is similar to that found for the frog liver enzyme (~2 s^{-1}; see above), but equilibration may occur more quickly when the products of the ATPase reaction leave the enzyme. It has been suggested (Sauers et al., 1975) that carbonyl phosphate in the enzyme may decompose reversibly to yield P_i and enzyme-bound CO_2, which would be the activated form of CO_2 that reacts with NH_3. If this were the case and if the P_i bound to the enzyme could rotate, incorporation of ^{18}O from $HC^{18}O_3$ into ATP_A as a consequence of reversal of the synthesis of enzyme-bound CO_2 should occur. The lack of ^{18}O labeling of ATP, therefore, argues against enzyme-bound CO_2 as an intermediate in the formation of carbamoyl phosphate (Figure 2).

Carbamoyl-phosphate synthetase from *E. coli* shows many differences from the rat liver enzyme. Glutamine is the preferred source of NH_3, acetylglutamate is not required for activation although other amino acids may activate and nucleotides are allosteric effectors, and there are also structural differences (Meister & Powers, 1978). Further, very different kinetics have been reported (Raushel & Villafranca, 1979; Elliott & Tipton, 1974). Nevertheless, in a recent study (Wimmer et al., 1979), positional isotope exchange gave a value of 0.63 for the ratio $V_{EX}/(V_H + V_{EX})$ similar to the value obtained in the present work, and incorporation of bicarbonate oxygen into P_i was found without incorporation into ATP. However, glutamine did not completely stop positional exchange [$V_{EX}/(V_H + V_{EX}) = 0.43$, 4 mM glutamine]. This last finding might be attributable to the hydrolysis of glutamine limiting the rate of supply of NH_3 at the active center. Thus, there is a considerable similarity in the positional isotope exchange data which suggests that the enzymes may share a common basic mechanism.

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Appendix: Kinetics of Incorporation of ^{18}O into ATP from $HC^{18}O_3$

The overall ATPase reaction in Figure 2 can be represented as

$$[^{18}O]ATP \underset{m_b}{\overset{m_a}{\rightleftharpoons}} \text{enzyme·}ADP_A\text{·}P_i\text{·}HC^{18}O_3\text{·}ATP_B \xrightarrow{m_{+3}} P_i$$

where m_a, m_b, and m_{+3} represent fluxes (Britton, 1966). These fluxes will be related to the fluxes in Figure 2 as follows:

$$m_a = m_{+1}m_{+2}/(m_{-1} + m_{+2}) \quad (1)$$

$$m_b = m_{-1}m_{-2}/(m_{-1} + m_{+2}) \quad (2)$$

Since only relative values are given in Figure 2, these values were adjusted to give the measured value of m_{+3}. The fractional ^{18}O content of HCO_3^- will fall exponentially because of exchange with water. Thus

$$a = a_0e^{-kt} \quad (3)$$

where a_0 and a are fractional ^{18}O contents of HCO_3^- at times = 0 and t; k = rate constant for exchange. If A^* represents the amount of ^{18}O labeled ATP, and A_0 the amount of ATP at $t = 0$, then

$$dA^*/dt = a_0m_be^{-kt} - m_aA^*/(A_0 - m_{+3}t) \quad (4)$$

Rearrangement of eq 4 yields

$$A^* = a_0m_b(A_0 - m_{+3}t)^{m_a/m_{+3}} \int_0^t (A_0 - m_{+3}t)^{-m_a/m_{+3}}e^{-kt}\,dt \quad (5)$$

The right-hand side of eq 5 was integrated numerically.

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Phospholipid Asymmetry in Semliki Forest Virus Grown on Baby Hamster Kidney (BHK-21) Cells†

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ABSTRACT: The distribution of the different phospholipid classes over the two leaflets of the membrane of Semliki Forest virus, grown on baby hamster kidney cells (BHK-21), was studied. To localize the phospholipids we have used a phosphatidylcholine-specific exchange protein, a nonspecific exchange protein, phospholipases A_2 and C, sphingomyelinase, and the amino group labeling reagent trinitrobenzenesulfonate. When low concentrations of exchange proteins and phospholipases were used, only those phospholipids present in lysed or otherwise defective virions were detected. Up to 10% of the phospholipids are present in such particles, and this amount correlated well with the amount of ribonuclease-degradable RNA present in each virus preparation. In the actual localization experiments, carried out with higher concentrations of exchange proteins and phospholipases as well as with trinitrobenzenesulfonate, the various independent techniques yielded identical results. The outer membrane layer of the virus contains 52% of the phosphatidylcholine, 22% of the phosphatidylethanolamine, and 33% of the sphingomyelin. Phosphatidylserine could not be localized. Altogether 30% of the total phospholipids could be assigned to the outer layer and 50% to the inner membrane leaflet whereas 20% could not be localized. Calculations, based on the size of the Semliki Forest virus and the number of phospholipid molecules per virion, indicate it to be unlikely that the phospholipids which could not be localized are present in the inner bilayer leaflet. The present data on phospholipid composition and distribution have been compared with previous results on the lipid distribution in plasma membrane derived structures. It is concluded that an asymmetric distribution of phospholipids in the plasma membrane of various mammalian cells is apparent.

An asymmetric distribution of the different phospholipid classes over the two membrane monolayers has been observed in a variety of biological membranes. The concept of lipid asymmetry has been best documented for plasma membranes from cells which do not contain additional intracellular membrane systems such as erythrocytes, bacteria, and viruses [for a review, see Op den Kamp (1979)]. Phospholipid localization studies on plasma membranes from nucleated eukaryotic cells are much more complicated and have been carried out mainly with derivatives of the plasma membrane. In general, two approaches have been followed to obtain plasma membrane derived structures.

Through the uptake of latex beads by mouse LM[1] cells, phagolysosomes are formed, which were considered to represent inside-out plasma membrane derivatives (Sandra & Pagano, 1978). Right side out derivatives of the plasma membrane can be studied by means of enveloped viruses which obtain their lipid constituents from the host cell plasma membrane during budding [for a review, see Patzer et al. (1979)]. The lipid composition of these viruses strongly resembles the lipid composition of the host cell plasma membrane. Extensive studies have been carried out with influenza virus (Tsai & Lenard, 1975; Rothman et al., 1976; Lenard & Rothman, 1976) and vesicular stomatitis virus (Patzer et al., 1978a,b; Shaw et al., 1979; Fong et al., 1976; Fong & Brown, 1978), which show that the phospholipids are distributed in an asymmetric way over the two halves of the virus membranes.

Also, Semliki Forest virus buds through the plasma membrane of the host cell (Acheson & Tamm, 1967; Richardson

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[1] Abbreviations used: SF virus, Semliki Forest virus; BHK-21, a baby hamster kidney cell line; LM, a fibroblast cell line; MDBK, Maden Darby bovine kidney; EDTA, ethylenediaminetetraacetic acid; TN, buffer containing 100 mM NaCl and 50 mM tris(hydroxymethyl)-aminomethane, pH 7.4, with HCl; TNBS, 2,4,6-trinitrobenzenesulfonate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; PI, phosphatidylinositol; $NaDodSO_4$, sodium dodecyl sulfate.

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