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# Alterations in the Energetics of the Carbamoyl Phosphate Synthetase Reaction by Site-directed Modification of the Essential Sulfhydryl Group*

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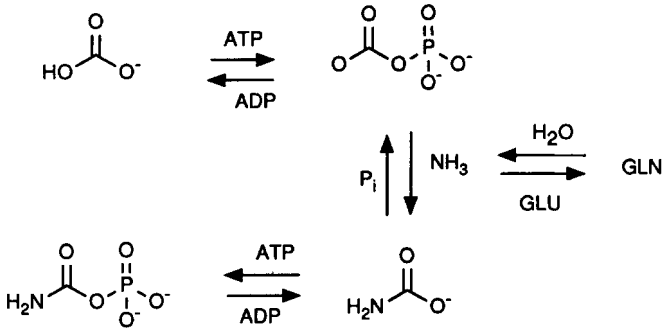
**The change in reaction energetics of the bicarbonate-dependent ATPase reaction of *Escherichia coli* carbamoyl phosphate synthetase has been investigated for two site-directed mutations of the essential cysteine in the small subunit. Cysteine 269 has been proposed to facilitate the hydrolysis of glutamine by the formation of a glutamyl-thioester intermediate. The two mutant enzymes, C269S and C269G, along with the isolated large subunit, exhibit a 2–2.6-fold increase in the bicarbonate-dependent ATPase reaction relative to that observed for the wild type enzyme. In the presence of glutamine the overall enhancement is 3.7 and 9.0 for the C269G and C269S mutant enzymes, respectively. Carboxyphosphate is an intermediate in the bicarbonate-dependent ATPase reaction. The cause of the rate enhancements was investigated by measuring the positional isotope exchange rate in [$\gamma$-$^{18}O_4$]ATP relative to the net rate of ATP hydrolysis. This ratio ($V_{ex}/V_{chem}$) is a measure of the partitioning of the enzyme-carboxyphosphate-ADP complex. The partitioning ratio for the mutants is identical within experimental error to that observed for the wild type enzyme. This observation is consistent with the conclusion that the ground state for the enzyme-carboxyphosphate-ADP complex in the mutants is destabilized relative to the same complex in the wild type enzyme. If the increase in the absolute rate of ATP hydrolysis was due to a stabilization of the transition state for carboxyphosphate hydrolysis then the positional isotope exchange rate relative to the chemical hydrolysis rate would have been expected to decrease in the mutants.**

Carbamoyl phosphate synthetase (CPS)[1] from *Escherichia coli* catalyzes the following reaction:

$$2\ \mathrm{MgATP} + \mathrm{HCO_3^-} + \mathrm{glutamine} + \mathrm{H_2O} \rightarrow \mathrm{carbamoyl\ phosphate} + 2\ \mathrm{MgADP} + \mathrm{P_i} + \mathrm{glutamate} \quad (1)$$

The carbamoyl phosphate that is formed in this reaction is further utilized in the pathways for both arginine and pyrimidine biosynthesis (Pierard and Wiame, 1964). This enzyme is of significant interest because of the large number of substrates, products, and allosteric effectors which interact to modulate the production of carbamoyl phosphate required for the two competing pathways.

CPS is a heterodimeric protein consisting of large (120 kDa) and small (42 kDa) subunits, (Matthews *et al.*, 1972). The reaction mechanism for the synthesis of carbamoyl phosphate consists of at least four individual steps and is illustrated in Scheme 1 (Powers and Meister, 1978; Raushel and Villafranca, 1979). Carboxyphosphate and carbamate are the two compounds that have been proposed as key intermediates in this reaction mechanism (Powers and Meister, 1978; Raushel and Villafranca, 1979).




SCHEME 1

The hydrolysis of glutamine to glutamate occurs on the small subunit while the remaining reactions take place on the large subunit (Trotta *et al.*, 1971). Therefore, the amide nitrogen of glutamine must be transferred to the active site of the large subunit where it can participate in the overall reaction. However, free ammonia can substitute for glutamine as the nitrogen source (Trotta *et al.*, 1971, 1974) for carbamoyl-P synthesis catalyzed by the large subunit.

Previous work has suggested that the mechanism for glutamine hydrolysis on the small subunit requires the participation of an essential sulfhydryl group for the formation of a thioester intermediate (Meister, 1989). Incubation of the enzyme with the glutamine analogs, L-2-amino-4-oxo-5-chloropentanoate, azaserine, and 6-diazo-5-oxonorleucine destroys the glutamine-dependent CPS activity while maintaining the ammonia-dependent activity (Khedouri *et al.*, 1966; Anderson *et al.*, 1973; Anderson and Carlson, 1975). Meister's laboratory also found that treatment of the enzyme with the chloroketone analog stimulated the bicarbonate-dependent ATPase reaction about 2-fold and decreased the apparent $K_m$ for ammonia (Khedouri *et al.*, 1966). The amino acid which reacts with the chloroketone was demonstrated by Pinkus and Meister (1972) to be a cysteine on the small subunit. These results were

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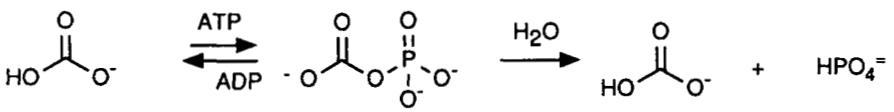
[1] The abbreviations used are: CPS, carbamoyl phosphate synthetase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2′-hydroxyethanesulfonic acid; PIX, positional isotope exchange.

confirmed by Rubino *et al.* (1987) when they utilized site-directed mutagenesis to change the critical cysteine 269 to either a glycine (C269G) or serine (C269S) residue. As expected, all of the glutamine-dependent synthesis of carbamoyl phosphate was lost, but the enzyme still retained the ability to use ammonia as the nitrogen source.

The C269S and C269G mutant enzymes were also reported by Rubino *et al.* (1987) to enhance the bicarbonate-dependent ATPase reaction 18- and 9-fold, respectively, relative to the wild type enzyme. The bicarbonate-dependent ATPase activity of the wild type enzyme results from the hydrolysis of the carboxyphosphate intermediate in the absence of a nitrogen source as shown in Scheme 2.




SCHEME 2

Normally, the bicarbonate-dependent ATPase reaction occurs at approximately 5% of the overall carbamoyl phosphate synthesis rate (Anderson and Meister, 1966). The slow rate of this partial reaction thus represents the ability of the enzyme to protect the unstable carboxyphosphate intermediate from $H_2O$. The isolation of the intermediate from $H_2O$ can result from either the exclusion of water from the active site and/or the very slow release of carboxyphosphate from the enzyme into the bulk solution in the absence of ammonia. However, it is not known whether carboxyphosphate is hydrolyzed on or off the enzyme. The two mutant enzymes constructed by Rubino *et al.* (1987) are unable to effectively exclude $H_2O$, and thus the reactions with $H_2O$ and $NH_3$ occur at comparable rates.

The energetics of the ATPase reaction catalyzed by the wild type enzyme have been examined in some detail by Raushel and Villafranca (1979) using rapid quench and positional isotope exchange experiments. The pre-steady-state time course for this reaction shows a burst of acid-labile phosphate followed by a significantly slower steady-state rate. These results demonstrated that the formation of enzyme-bound carboxyphosphate is very fast while the rate-limiting step involves either the actual attack by water on carboxyphosphate in the active site and/or product release. The kinetic barrier diagram (Burbaum *et al.*, 1989) for the simplified mechanism is illustrated in Scheme 3.

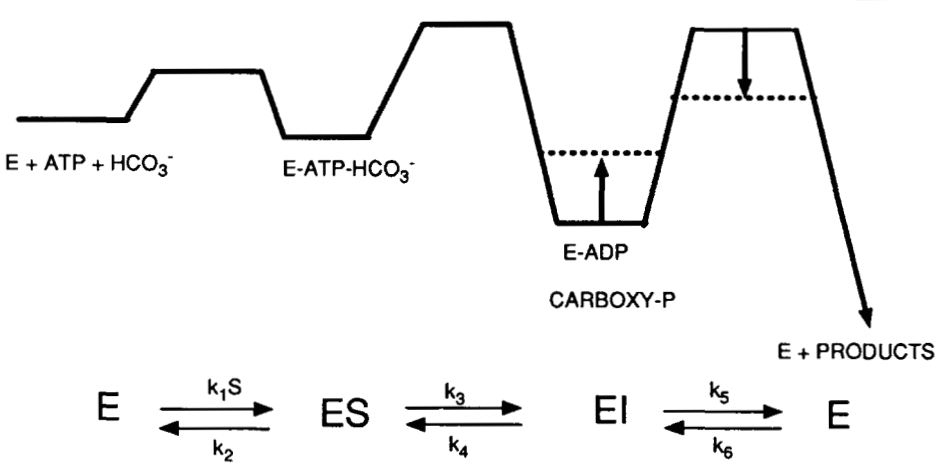
The C269S and C269G mutations offer the opportunity to determine how the energetics of the system have changed to accommodate the order of the magnitude increase in the reaction velocity of the ATPase reaction relative to the wild type enzyme. The increase in the reaction velocity can be accommodated in one of two ways. The transition state for the reaction of $H_2O$ with carboxyphosphate may be lowered in energy. The stabilization of the transition state for this step in the mechanism would increase the relative values for $k_5$ and $k_6$ by the same factor since the vertical distances in the kinetic barrier diagram are proportional to the logarithms of the reciprocal rate constants (Burbaum *et al.*, 1989). The value for $k_4$ would remain unchanged. Alternatively, the ground state for the enzyme-bound carboxyphosphate-ADP complex may be increased in energy. The destabilization of this complex would result in an increase in $k_4$ and $k_5$ by the same factor. These possibilities are graphically represented by the *vertical arrows* in Scheme III. In this paper we demonstrate how this distinction can be made by measuring the partitioning ratio of the *E*-carboxyphosphate-ADP complex utilizing positional isotope exchange technology.

## EXPERIMENTAL PROCEDURES

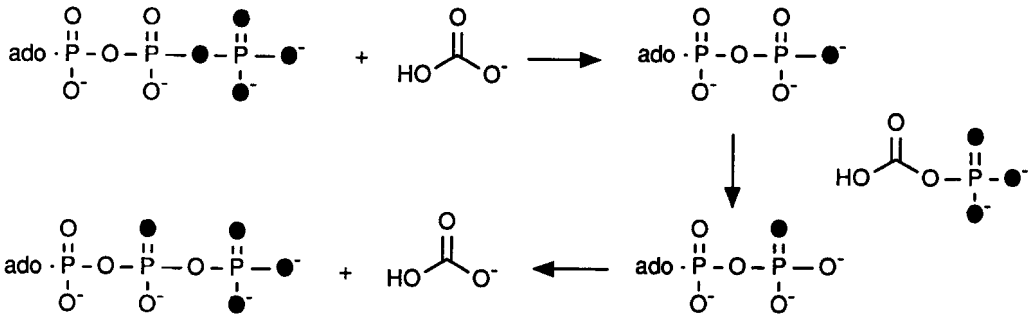
*Materials*—The plasmids containing the genes for the wild type enzyme (pMC41), the site-directed mutatations of the essential sulfhydryl group in the small subunit (C269S (pHN32) and C269G (pHN31)) and the isolated large subunit (pHN12) of carbamoyl phosphate synthetase were prepared as previously described (Rubino *et al.*, 1986, 1987). Oxygen 18-labeled water (97%) was purchased from Cambridge Isotope Laboratories. Oxygen 18-labeled potassium phosphate was made according to the procedure of Risley and Van Etten (1978). Preparation of [$\gamma$-$^{18}O_4$]ATP was made as described by Raushel and Villafranca (1980). DNase was purchased from Bethesda Research Laboratories. All other reagents were obtained from Sigma.

*Growth Conditions*—*E. coli* cells (strain RC50 (Mergeay *et al.*, 1974) were grown in TB media (12 g of Bactotryptone, 24 g of Bactoyeast, 4 ml of glycerol, 77 mM $K_2HPO_4$, 23 mM $KH_2PO_4$ in 1 liter) and harvested in stationary phase. The transformed cells were grown in the presence of 100 $\mu$g/ml carbenicillin.

*Purification of Wild Type and Mutant Proteins*—All of the enzymes were purified as described in Matthews and Anderson (1972) and Kaseman and Meister (1984) with minor modifications. Frozen cell paste (200 g) was suspended in 200 ml of 0.1 M potassium phosphate buffer, pH 7.5, 1.0 mM ornithine, 1.0 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (and 1.0 mM dithiothreitol for the isolated large subunit). The cells were lysed by sonication using a Heat




SCHEME 3



SCHEME 4

Systems Ultrasonics W-380 sonicator for 15 min at 90–100% full power with a 5-s cycle. After cell lysis, the suspension was treated with 1 mg of DNase and allowed to stir for 1 h at 4 °C and then centrifuged at 16,000 × *g* for 30 min. Solid ammonium sulfate was slowly added to the supernatant solution to a final concentration of 60% saturation. The suspension was centrifuged at 16,000 × *g* for 30 min. The pellet was then resuspended in 0.1 M potassium phosphate buffer, pH 7.5, 1.0 mM EDTA, and 40 mM $NH_4Cl$ and applied to an Ultrogel AcA34 column (5.0 × 154 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, 1.0 mM EDTA, and 40 mM $NH_4Cl$. The protein solution was eluted from the column with the same buffer. Those fractions containing active enzyme from the Ultrogel column were pooled and then applied to a Whatman DE52 anion exchange column equilibrated with 100 mM potassium phosphate, pH 6.8, and 1.0 mM EDTA. The CPS was eluted from this column with a 4.0-liter linear gradient starting from 100 mM potassium phosphate, pH 6.8, 1.0 mM EDTA and ending with 300 mM potassium phosphate, pH 7.6, 1.0 mM EDTA. Those fractions containing CPS were pooled, concentrated, and stored at −80 °C. Immediately prior to use the desired amount of enzyme was applied to a Waters DE5PW anion exchange high performance liquid chromatography column (2.2 × 15 cm) as the final purification step. The column was equilibrated with 100 mM HEPES, pH 7.5, and the protein chromatographed with a linear gradient of 1.0 M KCl in the same buffer. Carbamoyl phosphate synthetase eluted at approximately 200 mM salt. At this stage the proteins were greater than 90% pure as judged by sodium dodecyl sulfate-gel electrophoresis.

*Enzyme Assays*—Two assays were used to measure the activity of CPS. The first assay measured the rate of formation of citrulline by coupling the production of carbamoyl phosphate to ornithine transcarbamoylase. The assay contained 0.05 M HEPES, pH 7.6, 100 mM KCl, 20 mM $MgCl_2$, 40 mM $NaHCO_3$, 10 mM glutamine (or 300 mM $NH_4Cl$), 10 mM ornithine, 10 mM ATP, and 0.2 units of ornithine transcarbamoylase in a final volume of 0.25 ml. The reaction was quenched after specific time periods by the addition of 1.0 ml of an acid reagent followed by the addition of 0.5 ml of 0.4% diactyl monoxime. The acid reagent contained 3.7 g of antipyrine, 2.5 g of ferric ammonium sulfate in 500 ml of $H_2O$, 250 ml of $H_2SO_4$, and 250 ml of 85% $H_3PO_4$ (Snodgrass and Parry, 1969). The solution was vortexed and incubated at 100 °C in a water bath for 15 min. The citrulline concentration was determined from a standard curve using the absorbance at 464 nm and an extinction coefficient of 38 $mM^{-1}$ $cm^{-1}$.

The second assay measured the rate of formation of ADP catalyzed by CPS using the pyruvate kinase-lactate dehydrogenase coupled assay. The reaction mixtures contained 50 mM HEPES, pH 7.6, 1.0 mM PEP, 0.2 mM NADH, 15 mM glutamine (or 300 mM $NH_4Cl$), 40 mM $NaHCO_3$, 33 μg of lactic dehydrogenase, 33 μg of pyruvate kinase, and various amounts of CPS in a final volume of 3.0 ml. The decrease in absorbance was monitored at 340 nm.

*Positional Isotope Exchange Reactions*—The PIX reactions were followed by monitoring the interchange of the β-γ bridging oxygen to the β-nonbridging position (see Scheme 4) in [γ-$^{18}O_4$]ATP. CPS was incubated with 50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 100 mM KCl, 40 mM $HCO_3^-$, 10 mM ornithine, and 10 μmol of [γ-$^{18}O_4$]ATP in a final volume of 3.0 ml. Some reaction mixtures also contained 5 units of ornithine transcarbamoylase and either 10 mM glutamine or 300 mM $NH_4Cl$. The reaction was quenched by the addition of $CCl_4$ with vigorous vortexing and reduction of the pH to 2.5. After the enzyme was removed from the sample by passage through an Amicon CF-25 Centriflo filtration cone, the pH was raised to 9.0 and the volume reduced. The sample was then dissolved in 3.0 ml of a solution containing 100 mM EDTA, 50 mM potassium phosphate, 150 mM Tris, pH 9.0, and 25% $D_2O$. The positional isotope exchange rates were obtained from the $^{31}P$ NMR experiments as described previously (Raushel and Villafranca, 1980).

*$^{31}P$ Nuclear Magnetic Resonance Measurements*—$^{31}P$ NMR spectra were obtained on a Varian XL-400 multinuclear NMR spectrometer operating at a frequency of 162 MHz. Typical acquisition parameters were 6,000-Hz sweep width, 2.5-s acquisition time, no delay between pulses, 15-μs pulse width (90° pulse width = 25 μs), and Waltz decoupling (4 dB). All spectra were internally referenced to phosphate, pH 9.0.

## RESULTS

*Reactions Catalyzed by the Wild Type and Mutant Enzymes*—Table I summarizes the reactions catalyzed by the wild type enzyme and the mutants, C269S and C269G. The wild type enzyme can catalyze both glutamine and $NH_3$-dependent carbamyl phosphate synthesis at rates of 1,800 and 880 nmol/min-mg enzyme, respectively. However, the C269S and C269G mutant enzymes and the isolated large subunit are only able to catalyze the $NH_3$-dependent synthesis of carbamoyl phosphate. The rate of the $NH_3$-dependent synthesis catalyzed by the C269S and C269G mutant enzymes is comparable to the wild type enzyme indicating that the ability of the proteins to use $NH_3$ has not been affected by the single mutation of the essential sulfhydryl group of the small subunit.

The bicarbonate-dependent ATPase reaction catalyzed by the isolated large subunit and the C269S and C269G mutant enzymes is approximately 2-fold faster than the wild type enzyme. The C269G and C269S mutant enzymes exhibit an overall rate enhancement of 3.7- and 9.0-fold, respectively, for the ATPase reaction when glutamine is present. Glutamine has no effect on the rate of the bicarbonate-dependent

TABLE I

*Reactions catalyzed by the various CPS mutant enzymes*

| Reactions[a] | Wild type Cys-269 | Mutant Gly-269 | Mutant Ser-269 | Large subunit |
|---|---|---|---|---|
| Glutamine-dependent carbamoyl phosphate synthesis[b,c] | 1,800[d] | 0 | 0 | 0 |
| $NH_3$-dependent carbamoyl phosphate synthesis[b,e] | 880 | 820 | 670 | 340 |
| Bicarbonate-dependent ATPase[f] | 62 | 160 | 150 | 130 |
| Glutamine-enhanced bicarbonate-dependent ATPase[f,g] | NA[h] | 230 | 560 | 141 |

[a] 50 mM HEPES, pH 7.6, 100 mM KCl, 10 mM ornithine, 20 mM $MgCl_2$, 1.0 mM ATP, 40 mM $HCO_3^-$, and 27 °C.
[b] Measure rate of formation of carbamoyl phosphate.
[c] 10 mM glutamine.
[d] nmol/min-mg protein.
[e] 300 mM $NH_4Cl$.
[f] Measured rate of formation of ADP.
[g] 10 mM glutamine.
[h] NA, not applicable.

ATPase reaction of the isolated large subunit.

*Positional Isotope Exchange Reactions*—The results of the positional isotope exchange reactions are shown in Table II. The ratio of the positional isotope exchange rate to the chemical rate for net ATP hydrolysis ($V_{ex}/V_{chem}$) is essentially identical for all four enzymes. The ratio of the rate for the exchange reaction relative to the rate for net ATP hydrolysis of the C269S mutant enzyme is the same for the bicarbonate-dependent and glutamine-enhanced ATPase reaction. The upper limit for the ratio of the exchange rate relative to the net formation of carbamoyl phosphate from $NH_3$ is 0.10, 0.13, and 0.10 for the wild type, C269S mutant, and isolated large subunit enzymes, respectively.

## DISCUSSION

A simplified mechanism for the bicarbonate-dependent ATPase reaction of *E. coli* carbamoyl phosphate synthetase is shown in Scheme 3 where *E*S represents the enzyme-$HCO_3^-$-ATP complex and *E*I represents the enzyme-carboxyphosphate-ADP complex. At saturating levels of ATP and bicarbonate the rate of the reaction is governed by Equation 2 and the ratio of the rate for positional isotope exchange reaction and the rate for the net turnover of ATP ($V_{ex}/V_{chem}$) is given by Equation 3.

$$V_{max} = (k_3k_5)/(k_3 + k_4 + k_5) \quad (2)$$

$$V_{ex}/V_{chem} = (k_2k_4)/k_5(k_2 + k_3) \quad (3)$$

Previous isotope partitioning, PIX (Wimmer *et al.*, 1979), and rapid quench experiments with the wild type enzyme have demonstrated that $k_2$ is much larger than $k_3$ which is in turn much larger than either $k_4$ or $k_5$ (Raushel and Villafranca, 1979, 1980). The substantial rate enhancement for this reaction which is observed when the essential cysteine 269 of the small subunits is mutated to either serine or glycine has prompted us to examine the changes that have occurred in the reaction energetics.

The kinetic properties of the purified mutant enzymes are similar to those previously reported by Rubino *et al.* (1986, 1987). No glutamine-dependent synthesis of carbamoyl phosphate is observed for either the isolated large subunit or the C269S and C269G mutant enzymes thus confirming the essential features of cysteine 269. All three mutant enzymes catalyze an ammonia-dependent carbamoyl phosphate synthesis reaction. The bicarbonate-dependent ATPase reaction for the three mutant enzymes is 2–3-fold faster than the wild type enzyme, but upon the addition of glutamine the overall enhancement is increased by 4–10-fold. These values are somewhat smaller than those reported by Rubino *et al.* (1987).

TABLE II

*Positional isotope exchange reactions catalyzed by the various CPS mutants*

| Reaction[a] | $V_{ex}/V_{chem}$ | | | |
|---|---|---|---|---|
| | Cys-269 | Gly-269 | Ser-269 | Large subunit |
| Bicarbonate-dependent ATPase | 2.2 ± 0.7 | 2.9 ± 0.2 | 2.6 ± 0.2 | 1.8 ± 0.5 |
| Glutamine-enhanced ATPase[b] | | NA[c] | 2.7 ± 0.2 | |
| Carbamoyl phosphate synthesis[d] | <0.10 | NA[c] | <0.13 | <0.10 |

[a] Reaction conditions: 25 °C, 50 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 100 mM KCl, 40 mM $HCO_3^-$, 10 mM ornithine, 3.33 mM [$\gamma$-$^{18}O_4$]ATP.

[b] 10 mM glutamine added to reaction mixture.

[c] NA, not applicable.

[d] 5 units of ornithine transcarbamoylase and either 10 mM glutamine or 300 mM $NH_4Cl$ added to reaction mixture.

The significant increase in the bicarbonate-dependent ATPase reaction, especially the C269S enzyme in the presence of glutamine, must be the result of alterations in the rate constants $k_3$, $k_4$, and/or $k_5$. Since the rate-limiting step for the wild type enzyme involves the breakdown of enzyme-bound carboxyphosphate, $k_5$, then this rate constant must be increased. The mechanism for the change in this rate therefore involves either the stabilization of the transition state for carboxyphosphate hydrolysis and/or the destabilization of the ground state for the enzyme-bound carboxyphosphate complex. These limiting situations are depicted graphically in Scheme 3. The stabilization of the transition states for both the formation and breakdown of carboxyphosphate is highly unlikely since the rate of formation of carboxyphosphate has been shown to limit both the $NH_3$ and glutamine-dependent carbamoyl phosphate synthesis. No increase is observed in the $NH_3$-dependent carbamoyl phosphate rate with any of the mutants, and thus $k_3$ appears to remain unaltered with these mutant enzymes. If the transition state for carboxyphosphate hydrolysis is stabilized then only $k_5$ will be affected. Alternatively, if the enzyme-bound carboxyphosphate has been destabilized then both $k_4$ and $k_5$ will be increased by the same factor. These limiting cases can be differentiated by measurement of the partitioning rate of the *E*-ADP-carboxyphosphate complex. This is readily accomplished by measurement of the PIX rate relative to the net rate for ATP hydrolysis ($V_{ex}/V_{chem}$). This ratio is equivalent to $k_4/k_5$ since $k_2 \gg k_3$ (Equation 3). If the ground state for the enzyme-bound carboxyphosphate complex has been destabilized then the value for $V_{ex}/V_{chem}$ of the mutants will be identical to the wild type value since $k_4$ and $k_5$ will both be altered by the same factor. However, if only the transition state for carboxyphosphate hydrolysis is stabilized then the value for $V_{ex}/V_{chem}$ of the mutants will be substantially reduced since $k_5$ will now be significantly bigger than $k_4$.

The values for the ratio of the PIX rate and the net turnover of ATP are identical within experimental error for the wild type and three mutant enzymes. This is true for the bicarbonate-dependent ATPase reaction and for the glutamine-enhanced ATPase reaction of the serine 269 mutant. Therefore, it appears that alterations in the essential cysteine 269 of the small subunit result in the destabilization of the ground state for the enzyme-bound carboxyphosphate complex rather than a stabilization of the transition state for carboxyphosphate hydrolysis. This is true even when the ATPase reaction has been increased by one order of magnitude.

A different effect is observed with the wild type enzyme and the mutant enzymes when glutamine and/or $NH_3$ are added to the system to ultimately form carbamoyl phosphate. In every case examined the value for $V_{ex}/V_{chem}$ has been reduced over 20-fold, and thus the partitioning ratio for the enzyme-carboxyphosphate complex has been substantially altered. This demonstrates that $k_5$ has been increased significantly more than $k_4$. Therefore, the increased turnover in the presence of $NH_3$ results from the substantial stabilization of the transition state for the reaction of ammonia with carboxyphosphate relative to the reaction with water.

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