A Molecular Wedge for Triggering the Amidotransferase Activity of Carbamoyl Phosphate Synthetase[†]
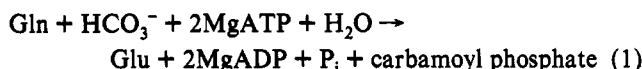
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ABSTRACT: The reactive cysteine residue within the small subunit of *Escherichia coli* carbamoyl phosphate synthetase has been identified using the technique of site-directed mutagenesis. Three cysteine residues have previously been found to react with *N*-ethylmaleimide (NEM) under controlled reaction conditions. Two of these cysteine residues are found on the large subunit, while the third cysteine is located on the small subunit. In the present investigation, Cys-248 of the small subunit has been identified as the residue that reacts with NEM in the presence of MgATP and bicarbonate. Three cysteine residues of the small subunit at positions 131, 214, and 248 were individually mutated to serine residues. These site-specific changes, in addition to *N*-ethylmaleimide-labeling studies, demonstrated that Cys-248 is the amino acid that reacts with *N*-ethylmaleimide. Substitution of Cys-248 of the small subunit with larger residues (Asp, Phe, Arg, and Trp) was conducted in order to more closely mimic the observed properties of the NEM-labeled enzyme. The partial glutaminase activity of the C248D mutant increased 40-fold relative to the wild-type enzyme, while the formation of carbamoyl phosphate using glutamine as a nitrogen source was completely abolished. Similar, but less dramatic, effects were observed for the other mutants, C248S, C248R, C248F, and C248W. There was good correlation between the extent of enhancement of the partial glutaminase activity and an uncoupling of the phosphorylation reactions that occur on the large subunit. These results have been interpreted to suggest that replacement of Cys-248 with bulkier residues induces the conformational changes in the small subunit that are thought to occur upon binding of MgATP and bicarbonate to the large subunit. The bulkier substituents serve as a molecular wedge for prevention of the native conformation and as a trigger for the enhancement of the hydrolysis of glutamine.

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

$$\text{Gln} + \text{HCO}_3^- + 2\text{MgATP} + \text{H}_2\text{O} \rightarrow$$
$$\text{Glu} + 2\text{MgADP} + \text{P}_i + \text{carbamoyl phosphate} \quad (1)$$

Ammonia can also be utilized as an alternate nitrogen source at high concentrations. The native enzyme is composed of two nonidentical subunits: a small subunit of 41 kDa and a large subunit of 118 kDa. The small subunit contains the amidotransferase activity, while the large subunit catalyzes the formation of carbamoyl phosphate from bicarbonate, ammonia, and MgATP (Piette et al., 1984; Nyunoya & Lusty, 1983). The large subunit also contains the binding sites for the allosteric effectors, IMP, UMP, and ornithine (Anderson & Meister, 1966; Trotta et al., 1971).

The catalytic activity of CPS previously has been found to be modified upon incubation with *N*-ethylmaleimide (NEM) (Anderson & Marvin, 1970). Foley et al. (1971) subsequently determined that three different cysteine residues within CPS are available for reaction with NEM and that these three cysteine residues can be sequentially labeled with this reagent under the appropriate reaction conditions. The first cysteine

residue is reactive with NEM under all conditions studied by Foley et al. (1971), and this is the only residue of CPS that reacts in the presence of ornithine. The labeling of this first reactive cysteine (E-NEM₁) does not affect the catalytic activity of the enzyme. In the presence of MgATP and bicarbonate, one additional cysteine residues reacts with NEM. The modification of this second cysteine residue (E-NEM₂) is accompanied by a 50-fold increase in the K_m for glutamine in the glutamine-dependent ATPase reaction[2] and 50% decreases in the carbamoyl phosphate synthetase and ATP synthesis activities of CPS. Moreover, the partial glutaminase activity is *increased* 20–30-fold (Wellner & Meister, 1974). The third cysteine residue reacts with high concentrations of NEM, and this reaction is facilitated by the presence of UMP. The labeling of the third reactive cysteine (E-NEM₃) results in almost complete loss of synthetase activity by the large subunit, but a 2-fold increase in the ATP synthesis activity relative to the native enzyme. Gel filtration chromatography, in the presence of guanidine hydrochloride, demonstrated that the first and third cysteine residues modified by NEM are located on the large subunit (Matthews & Anderson, 1972),

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[2] The catalytic activity of carbamoyl phosphate synthetase can be measured in a variety of assay formats. In this article, we will refer to the "glutamine-dependent ATPase reaction" when the rate of MgADP production is measured using an assay mixture containing MgATP, bicarbonate, and glutamine. The "ammonia-dependent ATPase reaction" follows the formation of MgADP when ammonia replaces glutamine as the nitrogen source. The terms, "glutamine- and ammonia-dependent carbamoyl phosphate synthetase reaction", are used when the rate of formation of carbamoyl phosphate is measured. In a fully coupled reaction, the rate of formation of MgADP should be twice the rate of formation of carbamoyl phosphate, as indicated by the reaction stoichiometry in eq 1.

while the second cysteine residue (labeled in the presence of MgATP and bicarbonate) is located on the small subunit.

In this report, we have conducted a more thorough investigation of the cysteine residue, located on the small subunit of CPS, that reacts with NEM in the presence of ATP and bicarbonate. We have selected this cysteine for further evaluation because this residue appears to reside at a site within the small subunit that undergoes a conformational change upon binding of ligands on the large subunit. Moreover, once this site is chemically modified, changes in the catalytic properties of both the large and small subunits are affected. Therefore, this residue can be utilized as a reporter group for the transmission of information between the active sites of the separate subunits. This information is critical for an understanding of the mechanism for translocation of ammonia between the subunit interface and the coordination of reactions occurring on the separate subunits.

There are four cysteines in the small subunit of CPS, and these residues are located at positions 131, 214, 248, and 269. Cys-269 has been previously shown to be critical for the hydrolysis of glutamine via the formation of a thioester intermediate, but it does not react with NEM (Wellner & Meister, 1974). The sequence identity of the NEM-reactive cysteine has now been determined by site-directed mutagenesis of the remaining cysteines to serine. The NEM-reactive sulfhydryl group has also been replaced by serine, asparate, tryptophan, phenylalanine, and arginine in an attempt to modulate the effects observed with NEM.

MATERIALS AND METHODS

Chemicals and Enzymes. Restriction enzymes were purchased from either Promega, Stratagene, or United States Biochemical Corporation. All chemicals and coupling enzymes were purchased from Sigma Chemical Company. The Sequenase kit was purchased from United States Biochemical Corporation. The Taq polymerase and the polymerase chain reaction (PCR) kit were purchased from Perkin-Elmer. NEM was obtained from Sigma, and [^{14}C]NEM was purchased from Amersham.

Bacterial Strains and Plasmids. The *E. coli* strains used in this study were RC50 (*carA*50, *thi*-1, *malA*1, *xyl*-7, *rsp*L135, λ^r, λ$^-$, *tsx*-273), XL1-Blue (*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsd*R17($r_k^-m_k^+$), *supE*44, *relA*1,λ$^-$, (lac), and [F', *proAB, lacI^q*ZM15, Tn10(*tet^r*)]) (Bullock et al., 1987; Kunkel et al., 1987; Joyce & Grindley, 1984). The strain RC50 was a generous gift from Dr. Carol J. Lusty (Public Health Research Institute of New York). The plasmid pDP412 was constructed as previously described (Post et al., 1990).

Construction of C131S, C214S, and C248S Mutant Enzymes. Site-directed mutations in the amidotransferase subunit (*carA*) of carbamoyl phosphate synthetase were constructed as described by Kunkel et al. (1987), and the resulting plasmids were transformed into *E. coli* strain XL1-Blue (*dut$^+$, ung$^+$*, F'). Transformant colonies were screened for the correct base changes by dideoxy sequencing of the denatured plasmids (Sanger et al., 1977) in a 200-bp region surrounding the mutation point. The mutated plasmids were transformed into the *carAB$^-$* strain RC50 for the expression and purification of mutant enzymes. The complementary oligonucleotides used to mutate the cysteine residues at amino acid positions 131, 214, and 248 of the small subunit are presented in Table 1. These primers, obtained from the DNA sequencing laboratory, Biology Department, Texas A&M University, introduced a single base change of G to C into the wild-type sequence.

Table 1: Site-Directed Changes in Carbamoyl Phosphate Synthetase[a]

plasmid	DNA and protein sequence	enzyme
pSM112	Gln Asn Gly Ser Ile Ile Ala Gly CAG AAT GGC TC*C ATT ATC GCG GGC G	C131S
pSM113	Leu Val Asp Arg Gly Ser Arg Leu Thr G GTG GAT AGA GGC TC*T CGC CTG ACC	C214S
pSM114	Pro Ala Pro Ser Asp Tyr Ala CCG GCC CCG TC*C GAT TAC GCC	C248S

[a] The oligonucleotides used were obtained from the DNA sequencing laboratory, Biology Department, Texas A&M University. These complementary primers introduce a single base change of G to C at indicated positions (*).

Table 2: Mutagenic Primers Used in the Site-Directed Changes at Position 248 in the Small Subunit of CPS[a]

plasmid	DNA sequence	enzyme
pSM120	5'G GCC CCG G*A*C GAT TAC GCC A3' 3'C CGG GGC C*T*G CTA ATG CGG T5'	C248D
pSM121	5'G GCC CCG TT*C GAT TAC GCC A3' 3'C CGG GGC AA*G CTA ATG CGG T5'	C248F
pSM123	5'G GCC CCG C*GC GAT TAC GCC A3' 3'C CGG GGC G*CG CTA ATG CGG T5'	C248R
pSM124	5'G GCC CCG TGG* GAT TAC GCC A3' 3'C CGG GGC ACC* CTA ATG CGG T5'	C248W

[a] These mutagenic primers introduce base changes at the indicated positions (*). The flanking primers used to isolate the mutant alleles were 5'CTT TTT GTC GCT TAA TGC CTG T and 5'CAC GCT TGC GCG CCA GAG T. The positions of the flanking primers within the *carA* gene are 270 and 1740, respectively.

Construction of C248D, C248R, C248F, and C248W Mutant Enzymes. Mutant alleles of *carA* encoding single amino acid substitutions for cysteine at position 248 were also constructed by site-directed mutagenesis of the pDP412 plasmid using the polymerase chain reaction (PCR) and the overlap extension protocol of Ho et al. (1989). The pairs of internal oligonucleotides encoding the mutations, together with the flanking primers, are listed in Table 2. The PCR-amplified mutagenic fragments were purified and digested with *Nsi*I and *Apa*I to facilitate insertion into the plasmid. The 1400-bp fragments generated by PCR were fully sequenced to confirm the desired mutations. The mutated plasmids were transformed into the *carAB$^-$* strain RC50 for the expression and purification of mutant enzymes.

Growth Conditions. All bacterial cells were grown in a modified Luria–Bertani (LB) broth (Maniatas et al., 1982) containing 24 g of yeast, 12 g of tryptone, 0.4% glycerol (per liter), and 0.1 M potassium phosphate. Cells were grown in the presence of 50 μg/mL ampicillin and harvested in stationary phase.

Purification of Wild-Type and Mutant Enzymes. The cells were lysed either by sonication or by passage through a French press (12 000 psi). EDTA (1.0 mM) and 0.2 mM phenylmethanesulfonyl fluoride (PMSF) were added to inhibit proteolysis, and then protamine sulfate was added dropwise to the lysate until a final concentration of 0.5% was reached. After centrifugation at 18000g for 20 min, the supernatant fluid was fractionated with 65% ammonium sulfate. The precipitate was resuspended in 0.1 M potassium phosphate buffer (pH 7.6) and 1.0 mM EDTA, loaded onto an Ultrogel AcA-34 column (5.0 × 200 cm), and then eluted with the same buffer. The fractions containing CPS were determined by enzymatic activity, sodium dodecyl sulfate–polyacrylamide

gel electrophoresis (SDS–PAGE), and absorbance at 280 nm. Pooled fractions were applied to a Waters Protein-Pak DEAE 5PW column (21.5 × 150 mm). CPS was eluted at a flow rate of 4 mL/min with a 100-mL gradient of 0.1 M potassium phosphate buffer (pH 7.6) containing 0–1.0 M KCl. The pooled fractions were stored at –70 °C until required.

Determination of Enzymatic Activity. Carbamoyl phosphate synthesis activity was determined by measuring the rate of citrulline formation at 25 °C in a coupled assay containing ornithine transcarbamylase (OTCase) and ornithine (Snodgrass et al., 1969). Each 0.5-mL reaction mixture contained 50 mM HEPES (pH 7.6), 2 mM ATP (pH 7.0), 100 mM KCl, 20 mM MgCl₂, 20 mM NaHCO₃, 10 mM glutamine, 5 mM ornithine and 0.4 unit of OTCase. Incubation at 25 °C for specified times was carried out upon addition of 10–40 μg of CPS. Reactions were quenched by adding an acid reagent and diacetyl monoxime (Rubino et al., 1986). The citrulline concentration was measured from the absorbance at 464 nm using an absorption coefficient of 37 800 M^{-1} cm^{-1} (Snodgrass et al., 1969).

The rate of ATP consumption was determined by measuring the amount of MgADP formed using a pyruvate kinase/lactate dehydrogenase (PK/LDH) coupling system. Each 2.5-mL reaction mixture contained 50 mM HEPES (pH 7.6), 1 mM phosphoenolpyruvate (PEP), 100 mM KCl, 0.2 mM NADH, 10 mM ornithine, 20 mM MgCl₂, 2 mM ATP (pH 7.0), 10 mM glutamine, 40 mM NaHCO₃, 20 units of PK, and 30 units of LDH. After addition of 10–40 μg of CPS, the reaction was monitored spectrophotometrically at 340 nm. For determination of the bicarbonate-dependent ATPase reaction, the formation of MgADP was measured using the PK/LDH coupling system, with the omission of glutamine from the reaction mixture.

The glutaminase activity of CPS was assayed by coupling the formation of L-glutamate to the production of α-ketoglutarate with L-glutamate dehydrogenase in a stepwise manner. The enzyme (20–40 μg) was incubated with 0.1 M CAPS (3-(Cyclohexylamino)-1-propanesulfonic acid, pH 10) and 20 mM L-glutamine at 25 °C in a volume of 400 μL. The reaction was stopped by the addition of 100 μL of 1 M HCl followed by a 15-min incubation on ice. The pH was neutralized by the addition of 100 μL of 1 M Tris (pH 10). This solution was then added to an assay mixture (600 μL) composed of 0.1 M Tris (pH 8.0), 0.8 mM acetylpyridine adenine nucleotide (APAD), and 10 units of L-glutamate dehydrogenase (GDH). The absorbance at 363 nm of the reduced APAD was measured after a 1-h incubation at 25 °C. The concentration of L-glutamate was calculated from a standard curve established with known amounts of L-glutamate.

Kinetic data were fit to eq 2 using the computer programs obtained from Sahara Shell Software. In this equation, v is the initial velocity, V_{max} is the maximal velocity, K_m is the Michaelis constant, and [S] is the substrate concentration:

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

NEM Labeling of Reactive Cysteine. For derivatization of the cysteine residue located on the small subunit of CPS with NEM, the more reactive cysteine residue on the large subunit was initially labeled using the procedure of Matthews and Anderson (1972). In a volume of 3 mL, carbamoyl phosphate synthetase (5–10 mg) was incubated with 0.13 M potassium phosphate (pH 7.0), 13 mM ornithine, 0.3 mM

EDTA, and 0.38 mM NEM for 1 h at 17 °C. To quench the reaction, 250 μL of 50 mM β-mercaptoethanol was added. Excess NEM was removed using PD-10 columns containing Sephadex G-25M obtained from Pharmacia. In a volume of 1 mL, 4–6 mg of the derivatized enzyme was then incubated with 10 mM ATP, 10 mM MgCl₂, 10 mM bicarbonate, and 0.4 mM [¹⁴C]NEM for 2 h at 17 °C. The reaction was again quenched with β-mercaptoethanol, and excess [¹⁴C]NEM was subsequently removed by dialysis against 0.1 M potassium phosphate (pH 7.6). Radioactivity measurements of the labeled enzyme were carried out using a Beckman LS 6000SE scintillation counter.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) Analysis. SDS–PAGE was utilized to separate the two subunits for individual radioactivity measurements. A 10% gel was run as described by Laemmli (1970), and the locations of the subunits were identified by matching the gel with a stained gel containing a control CPS sample. Portions of the gel containing the protein were excised, and the protein was eluted from the gel by incubation in 0.5 mL of 0.1 M potassium phosphate (pH 7.6) for at least 48 h.

RESULTS

Construction and Purification of Site-Directed Carbamoyl Phosphate Synthetase Mutants. The cysteine residues at positions 131, 214, and 248 of the small subunit of CPS were individually replaced with serine residues using the mutagenesis protocol of Kunkel et al. (1987). In addition, the cysteine residue at position 248 was also changed to aspartic acid, arginine, phenylalanine, and tryptophan using PCR methodology (Ho et al., 1989). The resulting plasmids were transformed into the *car*AB⁻ strain RC50 for the expression and subsequent purification of the mutant enzymes. The wild-type carbamoyl phosphate synthetase and the seven individual mutant proteins were purified to apparent homogeneity. All of the mutated proteins appeared to be as stable as the wild-type enzyme. Physical association of the two subunits making up the heterodimer was verified by the activity and protein profiles obtained during the gel filtration step of the purification procedure. A single elution peak, corresponding to the intact holoenzyme, was obtained during each of the purifications.

Kinetic Properties of C131S, C214S, and C248S Mutant Enzymes. The kinetic parameters, V_{max} and K_m, for the wild-type and cysteine mutant enzymes are presented in Table 3. The kinetic constants have been obtained for the glutaminase activity, the glutamine-dependent ATP hydrolysis activity, and the carbamoyl phosphate synthetase activity. All reactions were carried out in the presence of the allosteric effector, ornithine. Modest, but statistically significant, changes are noted for the mutant C248S. A 5-fold increase in the Michaelis constant for glutamine is obtained for the glutamine-dependent ATPase reaction, and a 2-fold enhancement of the V_{max} for the glutaminase reaction is observed with the C248S mutant. Much smaller effects are observed for the C131S and C214S mutant enzymes. Of the three mutant enzymes, C248S exhibits characteristics parallel to those obtained from the previous NEM-labeling studies (Foley et al., 1971; Wellner & Meister, 1974). However, the changes in the glutaminase activity are much lower than those previously observed with the NEM-labeled native enzyme.

N-Ethylmaleimide Labeling Studies of C131S, C214S, and C248S Mutant Enzymes. Labeling studies using [¹⁴C]-NEM were carried out with the mutant enzymes, C131S, C214S, and C248S, in order to provide further support for the

Table 3: Kinetic Parameters of the Wild-Type and Mutant Enzymes

	reaction monitored					
	glutamine-dependent		glutamine-dependent CP synthesis[a]		glutaminase[b] (pH 9.5)	
enzyme	V_{max} (μmol/min·mg)	K_{Gln} (mM)	V_{max} (μmol/min·mg)	K_{Gln} (mM)	V_{max} (μmol/h·mg)	K_{Gln} (mM)
WT	4.0 ± 0.2	0.11 ± 0.01	1.5 ± 0.1	0.21 ± 0.02	2.7 ± 0.1	1.8 ± 0.2
C131S	2.0 ± 0.1	0.05 ± 0.01	1.0 ± 0.04	0.16 ± 0.02	1.5 ± 0.1	1.4 ± 0.3
C214S	2.5 ± 0.1	0.07 ± 0.01	1.1 ± 0.02	0.13 ± 0.01	2.3 ± 0.3	1.8 ± 0.6
C248S	1.8 ± 0.1	0.51 ± 0.06	1.3 ± 0.08	0.99 ± 0.13	5.1 ± 0.2	1.5 ± 0.1

[a] pH 7.6, 25 °C, variable glutamine, 40 mM bicarbonate, 20 mM Mg^{2+}, and 2 mM ATP. [b] pH 9.5, 25 °C, variable glutamine, 40 mM bicarbonate, 20 mM Mg^{2+}, and 2 mM ATP.

Table 4: Quantitation of Radioactivity on the Derivatized Wild-Type and Mutant Enzymes[a]

		fraction of each subunit labeled	
enzyme	CPM/mg or protein	large subunit	small subunit
WT	63 700	0.06	0.80
C131S	96 200	0.14	1.22
C214S	81 600	0.15	1.00
C248S	39 600	0.36	0.20

[a] Cold NEM was used to label the residue that reacts under all conditions studied by Foley et al. (1971). [^{14}C]NEM was then used to label the cysteine group on the small subunit, which reacts in the presence of MgATP and bicarbonate.

conclusions based on the results from the measurement of the kinetic constants. With each mutant protein, non-radioactive NEM was used to label the cysteine residue on the large subunit that will react under all experimental conditions. Subsequent labeling of the NEM-modified enzyme in the presence of MgATP and bicarbonate was then conducted using [^{14}C]-NEM. Under these conditions, the reactive cysteine residue on the small subunit is predominantly modified with the radioactive label, while the reactive cysteine on the large subunit will be labeled with cold NEM. Samples of the derivatized mutant enzymes were then analyzed for radioactivity after dialysis to remove excess NEM.

Identification of the location of [^{14}C]NEM was performed using SDS–polyacrylamide gel electrophoresis. The two subunits were separated by electrophoresis, and gel slices of the protein bands were excised and counted for the presence of radioactivity. The results are presented in Table 4, and they indicate that most of the NEM labeling of the wild-type, C131S, and C214S enzymes is located on the small subunit. The stoichiometry of labeling for the small subunit of these enzymes varies between 0.8 and 1.2, while the stoichiometry of labeling for the large subunit is less than 0.15. In contrast, most of the radioactive label on C248S is found on the large subunit. The stoichiometry of labeling for the large and small subunits is 0.36 and 0.20, respectively. These results imply that the reactive cysteine residue located on the small subunit of the wild-type protein is no longer available for reaction with NEM with this mutant. The cysteine residue at position 248 of the small subunit is therefore involved in the specific reaction with NEM in the presence of MgATP and bicarbonate.

Kinetic Studies of C248D, C248F, C248R, and C248W Mutant Enzymes. The results presented above suggest that the cysteine residue at position 248 of the small subunit reacts with NEM in the presence of MgATP and bicarbonate. To further investigate this site of CPS, bulkier substitutions were carried out at this position, and the effects of these changes were analyzed. The kinetic parameters for the wild-type and mutant enzymes are presented in Tables 5 and 6. The K_m values for glutamine in the glutamine-dependent ATP hydrolysis activity showed significant increases relative to the

native enzyme. The C248W enzyme exhibits the largest increase, with a K_m value close to 250 mM compared to 0.11 mM for the wild-type enzyme. The catalytic activity of the carbamoyl phosphate synthetase activity of C248W decreased significantly, thus indicating a decrease in the catalytic efficiency of this mutant enzyme toward carbamoyl phosphate formation. It is interesting to note that the rate of formation of carbamoyl phosphate with this mutant is significantly lower in the presence of ammonia (Table 5). The C248D enzyme is unable to synthesize carbamoyl phosphate with glutamine, but is able to do so in the presence of ammonia.

The effects of the site-directed changes on the glutaminase activity are shown in Table 6. The glutaminase activity was measured at pH 7.6 and 9.5. The activities of the mutant enzymes at both pH values are all significantly higher than the wild-type activity. Catalytic activities are also observed to be higher at pH 9.5 compared to those measured at pH 7.6.

DISCUSSION

The initial objective of this study was to identify the NEM-reactive cysteine residue of the small subunit of CPS that causes the substantial rate enhancement for the hydrolysis of glutamine. Of the four cysteine residues within the small subunit of CPS from *E. coli*, only one (Cys-269) is totally conserved in the amino acid sequence when compared to other *trp*G-type amidotransferase enzymes and CPS enzymes from other sources. Cysteine-269 previously has been found to be directly involved in the chemical mechanism for glutamine hydrolysis, but prior experiments have demonstrated that this residue does not react with NEM under any conditions (Wellner & Meister, 1974). The other cysteine residues at positions 131, 214, and 248 have now been mutated to other amino acids, and the effects of these changes on the kinetic parameters exhibited by the mutated enzymes have been analyzed.

The kinetic properties for two of the mutants constructed in this study, C131S and C214S, are altered only slightly when compared to those of the unmodified native enzyme. These two mutants utilize glutamine in the full biosynthetic reaction and in the partial glutaminase reaction as well as the wild-type enzyme. It therefore appears that the cysteine residues at either of these two positions are not critical for the binding and hydrolysis of glutamine. However, replacement of the cysteine at position 248 with a serine residue results in a modest increase in the glutaminase reaction rate. Moreover, this mutation is also accompanied by a 5-fold increase in the K_m for glutamine. However, the overall changes in the kinetic constants with this mutant enzyme are not as dramatic as when the native enzyme is labeled with NEM in the presence of MgATP and bicarbonate (i.e., a 20–30-fold increase in the glutaminase activity and a 50-fold increase in the K_m for glutamine for the glutamine-dependent ATP hydrolysis activity).

Table 5: Kinetic Parameters of the Wild-Type and Mutant Enzymes

	reaction monitored								
	Gln-dependent ATPase[a]		NH4+-dependent ATPase[b]		Gln-dependent CP synthesis[a]		NH4+-dependent CP synthesis[b]		HCO3−-dependent ATPase[c] Vmax (μmol/min·mg)
enzyme	V_{max} (μmol/min·mg)	K_{Gln} (mM)	V_{max} (μmol/min·mg)	$K_{NH_4^+}$ (mM)	V_{max} (μmol/min·mg)	K_{Gln} (mM)	V_{max} (μmol/min·mg)	$K_{NH_4^+}$ (mM)	
WT	4.0 ± 0.2	0.11 ± 0.01	2.4 ± 0.2	180 ± 40	1.5 ± 0.1	0.21 ± 0.02	0.5 ± 0.02	190 ± 10	0.075
C248S	1.8 ± 0.1	0.5 ± 0.1	0.97 ± 0.08	200 ± 40	1.3 ± 0.08	0.99 ± 0.13	0.3 ± 0.02	440 ± 20	0.012
C248D	≤0.03[d]		0.7 ± 0.03	220 ± 30	≤0.03		0.2 ± 0.01	260 ± 20	0.024
C248F	2.3 ± 0.3	17 ± 4	0.4 ± 0.03	220 ± 30	0.9 ± 0.02	21.5 ± 0.8	0.4 ± 0.04	440 ± 80	0.008
C248R	2.0 ± 0.04	7.4 ± 0.3	1.5 ± 0.1	160 ± 20	1.1 ± 0.03	10.8 ± 0.6	0.4 ± 0.02	390 ± 20	0.035
C248W	0.5 ± 0.1	252 ± 60	0.06 ± 0.003	140 ± 20	0.08 ± 0.02	114 ± 34	0.02 ± 0.003	460 ± 100	0.005

[a] pH 7.6, 25 °C, variable glutamine, 40 mM bicarbonate, 20 mM Mg^{2+}, and 2 mM ATP. [b] pH 7.6, 25 °C, variable ammonium chloride, 40 mM bicarbonate, 20 mM Mg^{2+}, and 2 mM ATP. [c] pH 7.6, 40 mM bicarbonate, 20 mM Mg^{2+}, and 2 mM ATP. [d] There was no difference in the rate of ADP formation when either glutamine or ammonia was added to reaction mixtures containing CPS, MgADP, and bicarbonate.

Table 6: Kinetic Parameters for the Glutaminase Reaction of the Wild-Type and Mutant Enzymes

	pH 7.6		pH 9.5	
enzyme	V_{max} (μmol/h·mg)	K_m (mM)	V_{max} (μmol/h·mg)	K_m (mM)
WT	0.6 ± 0.04	1.2 ± 0.2	2.7 ± 0.1	1.8 ± 0.2
C248S	3.2 ± 0.1	0.52 ± 0.07	5.1 ± 0.1	1.4 ± 0.3
C248D	44 ± 0.9	7.2 ± 0.5	105 ± 7	30 ± 4
C248F	12 ± 0.2	0.91 ± 0.04	18 ± 0.5	2.0 ± 0.2
C248R	4.5 ± 0.1	1.4 ± 0.3	16 ± 0.5	3.2 ± 0.4
C248W	24 ± 0.3	2.7 ± 0.1	38 ± 2.6	6.1 ± 1.1

Labeling studies with [14C]NEM have confirmed the involvement of Cys-248 as the site of reaction with this reagent. It has been shown that C131S, C214S, and the wild-type enzyme are preferably labeled with NEM on the small subunit of CPS in the presence of MgATP and bicarbonate (Table 4). A minimum stoichiometry of 0.8 for labeling of the small subunit has been demonstrated with the abovementioned enzymes, and less than 15% of the large subunit is labeled. Much lower levels of overall labeling are detected with the C248S mutant enzyme, and a majority of the radioactivity is demonstrated to be associated with the large subunit.

The covalent attachment of NEM to a cysteine residue is expected to cause a conformational distortion of the protein structure in the vicinity of the labeling site on the basis of the observed effects on the kinetic properties. Therefore, four new mutant proteins with bulkier substituents at position 248 were constructed in order to generate mutants that would more closely mimic the kinetic properties of the NEM-labeled protein. The four new mutants, C248D, C248F, C248R, and C248W, produce significantly greater changes in the kinetic properties than does C248S, when compared to the wild-type enzyme. The substantial increases in the partial glutaminase reaction and the decreases in the carbamoyl phosphate synthetase reaction rates (Tables 5 and 6) suggest that conformational changes are induced in both subunits upon mutation of the small subunit at position 248 with these bulkier side chains. The observed kinetic parameters of these mutants are comparable to those previously obtained with the NEM-labeling studies.

The C248D mutant has totally lost the ability to generate carbamoyl phosphate from glutamine. However, the ammonia-dependent carbamoyl phosphate synthetase activity is maintained but at a slightly lower rate. Thus, the hydrolysis of glutamine and the synthesis of carbamoyl phosphate have become totally uncoupled with this mutant. The uncoupling of the reactions occurring at each active site is complete since the binding of glutamine on the small subunit can no longer stimulate the hydrolysis of ATP on the large subunit (see

Table 5). The C248W mutant displayed a significant decrease in the rate of formation of carbamoyl phosphate (Table 5). Not only is the translocation of ammonia from the small subunit hindered but, in addition, the utilization of ammonia for the synthesis of carbamoyl phosphate is also adversely affected. The conformational change resulting from this particular mutation seems to hinder the formation of carbamoyl phosphate.

The enzymatic synthesis of carbamoyl phosphate is a complex process. The phosphorylation reactions involving ATP occur on the large subunit, while the hydrolysis of glutamine for the generation of ammonia occurs on the small subunit. The physical distance separating these sites is unknown, as are many of the details concerning the mechanism for translocation of ammonia from the site of generation to the site of utilization. Moreover, the reactions that occur on the separate subunits must be synchronized for optimal utilization of substrates at either site. With the wild-type enzyme, the partial reactions are in fact quite slow (see Tables 3 and 5 for the observed partial glutaminase- and bicarbonate-dependent ATPase reaction rates relative to the overall synthesis of carbamoyl phosphate). Net turnover is stimulated substantially by the addition of the full complement of substrates, and thus the binding of substrates to one subunit must be communicated to the other subunit by induced conformational changes.

Physical evidence for the conformational changes induced by substrates bound to CPS has been obtained. Raushel et al. (1979) demonstrated that the mobility of a nitroxide spin label attached to the small subunit is increased upon binding of ATP to the large subunit. Anderson and Carlson (1975) also showed that the binding of MgATP and bicarbonate stimulates the reaction of cyanate with the active-site thiol (Cys-269) on the small subunit. In addition, mutation of Glu-841 in the large subunit affects the glutaminase activity on the small subunit (Lusty & Liao, 1993). And finally, Cys-248 is reactive with NEM only after MgATP and bicarbonate are bound to the large subunit and, thus, it appears that this particular sulfhydryl group becomes exposed only upon binding of these substrates to the large subunit.

The conformational change that occurs within the small subunit of CPS upon binding of MgATP and bicarbonate to the large subunit with the native enzyme can be locked into place by reaction of the exposed sulfhydryl group in the small subunit with an NEM label. This molecular wedge can prevent the small subunit from relaxing to the initial structure, and thus the labeled subunit remains fully activated for glutamine hydrolysis. The effects induced by NEM can be closely mimicked by the substitution of amino acids with bulky side

chains for the native cysteine at this position. The most extreme case is with the mutant C248D. The partial glutaminase reaction is stimulated by nearly 2 orders of magnitude, but the ammonia is unable to be effectively used by the large subunit for the synthesis of carbamoyl phosphate.

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