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Effect of Growth Temperature on Folding of Carbamoylphosphate Synthetases of *Salmonella typhimurium* and a Cold-Sensitive Derivative

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The properties of homogeneous preparations of carbamoylphosphate synthetase (CPSase) from wild-type *Salmonella typhimurium* and a cold-sensitive derivative grown at different growth temperatures were examined. For the cold-sensitive mutant, the affinity for glutamine of the form of CPSase synthesized at 20°C was lower than that of the form of the enzyme synthesized at 37°C, regardless of the assay temperature. Thus, the cold sensitivity of the mutant reflects an effect of temperature on the synthesis of the enzyme rather than the activity of the folded enzyme. The two forms also differed in sensitivities to polyclonal antibodies as well as denaturational enthalpies. The combined results support the hypothesis that *carAB* mutations conferring cold sensitivity identify amino acid residues that are critical in the folding of CPSase. Quite unexpectedly, certain kinetic properties of cloned parent CPSase were also dependent on the growth temperature, although to a much lesser extent than those of the cold-sensitive mutant. The specific activity of wild-type CPSase synthesized at 15°C was 60% of that synthesized at 37°C. Further, CPSase synthesized at 15°C was less thermostable than the enzyme synthesized at 37°C; the difference in stability (ΔG) is estimated to be 4,500 cal mol^{-1}. Thus, variation of temperature within the physiological range for growth influences the folding and consequently the properties of CPSase from wild-type *S. typhimurium*.

Carbamoylphosphate is an intermediate in the biosynthesis of both arginine and pyrimidines. In *Salmonella typhimurium* (1–3), a single enzyme, carbamoylphosphate synthetase (EC 6.3.5.5) (CPSase) catalyzes the synthesis of carbamoylphosphate according to the following reaction:

$$2\text{MgATP} + \text{HCO}_3^- + \text{L-glutamine} + \text{H}_2\text{O} \xrightarrow[\text{Mg}^{2+}]{\text{K}^+} \text{Carbamoylphosphate} + \text{P}_i + 2\text{MgADP} + \text{L-glutamate}$$

Ammonia can replace glutamine as a nitrogen donor in vitro, but the affinity for ammonia is considerably lower than that for glutamine; the latter substrate is the physiological nitrogen donor.

The enzyme consists of two nonidentical subunits (2). The light subunit (α; M_r, 45,000), encoded by *carA*, functions as a glutamine amidotransferase, and the large subunit (β; M_r, 110,000), encoded by *carB*, mediates all other catalytic functions. The large subunit alone can catalyze the synthesis of carbamoylphosphate from ammonia, bicarbonate, and MgATP (2). In accordance with its physiological function, the expression of the *carAB* operon is subject to control by arginine and pyrimidines (19, 23). Further, CPSase activity is subject to allosteric control by intermediates of pyrimidine and arginine biosynthesis: UMP inhibits the reaction, whereas ornithine stimulates it (3). The combined effects provide an efficient mechanism for controlling the rate of synthesis of carbamoylphosphate in response to the availability of arginine and pyrimidines. The enzyme also is activated by IMP and phosphoribosyl-1-pyrophosphate, effects which presumably coordinate its activity with purine biosynthesis (3).

Null mutations in *carA* and *carB* cause auxotrophy for both arginine and pyrimidines. However, certain missense mutations in these two genes cause auxotrophy only for arginine in *S. typhimurium* (1) as well as in *Escherichia coli* (24). The high frequency of this arginine auxotrophic phenotype of *carA* and *carB* resulted in early mismapping of the structural gene for ornithine carbamoyltransferase in *S. typhimurium* (33) and *E. coli* B/r (4).

We have shown previously (1) that the arginine auxotrophic phenotype of *carAB* is often more marked at low temperature (20°C). Genetic and physiological studies (1) with such cold-sensitive arginine auxotrophs suggested that the inability of such mutants to grow at the restrictive temperature resulted from defective folding of CPSase. Here we report studies with homogeneous CPSase preparations from one of these cold-sensitive mutants showing that the mutant at 20°C is a stable folding variant of the enzyme that differs in kinetic, calorimetric, and immunological properties from the form synthesized at 37°C.

The present report also describes the unexpected finding that the growth temperature of wild-type *S. typhimurium* influences the folding of CPSase. The two forms synthesized at 37 and 15°C differ in certain kinetic properties, and the form synthesized at low temperature has lower thermal stability.

MATERIALS AND METHODS

Culture and culture conditions. *S. typhimurium* AA222 (*carAB81 pyrB*::Tn*10*) carrying the *carAB* operon on a multicopy plasmid (pMK1 [19]) was grown in glucose minimal medium (33) supplemented with uracil (50 μg/ml) and used as a source for wild-type CPSase. *S. typhimurium*

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JL2108 (*pyrA1510*) was isolated earlier by the mutagenized bacteriophage technique (1).

Mapping of *pyrA1510*. The mutation was mapped by using reciprocal three-factor crosses with the cotransducing marker *fol*, which confers resistance to trimethoprim and is 15% linked to *carAB* in crosses mediated by phage P22 HT105 (31). The *pyrA1510* mutation mapped between *pyrA78* and *pyrA102*, both of which are deletions in *carB* (3). Accordingly, *pyrA1510* was redesignated as *carB1510*.

CPSase assay. CPSase activity was assayed by the incorporation of [^{14}C]bicarbonate into carbamoylphosphate as previously described (3). Reaction mixtures (final volume, 0.5 ml) contained enzyme, 100 mM triethanolamine buffer (pH 8.0), 12 mM ATP, 16 mM $MgCl_2$, 10 mM $NaH^{14}CO_3^-$, 100 mM KCl, and 10 mM glutamine or 100 mM NH_4Cl. For kinetic studies, enzyme concentration and reaction time were selected such that less than 5% of the variable substrate was consumed. Under these conditions, the reaction rates were linear for 15 min and were proportional to enzyme concentration over a 20-fold range of concentration. One unit of CPSase activity catalyzes the formation of 1 nmol of carbamoylphosphate per min.

Purification of CPSase. Exponentially growing cells were harvested by filtration with the Pellicon Cassette System (Millipore Corp., Bedford, Mass.), washed in 0.1 M potassium phosphate (pH 7.6) containing 1.0 mM EDTA, and suspended at 0.5 g (wet weight) per ml in the same buffer. All solutions coming in contact with the enzyme in subsequent steps contained 1.0 mM EDTA. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM immediately before the passage of the cell suspension through an Aminco French pressure cell at 8,000 lb/in^2. The crude extract was centrifuged at 27,000 × *g* for 30 min. Streptomycin sulfate (1 ml of 20% solution per 10 ml of centrifuged extract) was added at 0°C with stirring and equilibrated for 10 min. After centrifugation at 27,000 × *g* for 30 min at 4°C, the supernatant was pumped at a rate of 6 ml/min onto a preparative Q Sepharose (Pharmacia, Inc., Piscataway, N.J.) column (70 by 2.6 cm) equilibriated with 0.1 M potassium phosphate (pH 7.6). The protein was eluted with a linear gradient of potassium chloride in 0.1 M potassium phosphate (pH 7.6). CPSase eluted between 0.56 and 0.67 M KCl. Fractions containing CPSase were combined, concentrated with ammonium sulfate (70% saturation), and dissolved in a minimal volume of 20 mM potassium phosphate buffer (pH 7.6). The solution was subjected to gel filtration on a preparative Superose 12 (Pharmacia) column (50 by 1.6 cm) equilibriated with 20 mM potassium phosphate (pH 7.6) and eluted with the same buffer. The retention volume was 40 ml. Fractions containing CPSase were combined and filtered through a Millipore membrane (0.45-μm pore size) and pumped at a rate of 8 ml/min onto a Pharmacia Mono Q column (HR 16/10) equilibrated with 20 mM potassium phosphate (pH 7.6). After being washed with 100 ml of this buffer, protein was eluted with a linear gradient of KCl. CPSase activity was eluted between 0.25 and 0.35 M KCl. Fractions containing CPSase were combined, precipitated with ammonium sulfate (70% saturation), and centrifuged at 12,000 × *g* for 20 min. The precipitate was dissolved in a minimal volume of 0.1 M potassium phosphate buffer (pH 7.6), and the solution was applied onto a Pharmacia Superose 6 column (HR 10/30) and eluted with 0.1 M potassium phosphate (pH 7.6) at a rate of 0.3 ml/min. The elution volume for CPSase was 13.6 ml.

CPSases from the wild-type parent and the cold-sensitive derivative exhibited identical retention volumes during gel filtration. Further, they were eluted at the same KCl concentration during chromatography on the Mono Q anion exchanger, indicating that the surface charges of all the preparations from the parent strain and the cold-sensitive derivative were similar at pH 7.6. All preparations used in kinetic and calorimetric studies were homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10 to 15% gradient gels (Pharmacia Phast System). Table 1 shows the specific activities for the purified preparations.

TABLE 1. Specific activities of purified CPSase preparations

Strain (genotype)	Growth temp (°C)	Sp act[a] (nmol/min per mg)
AA222(pMK1) (wild type)	37	2,496.7
	20	2,425.8
	15	1,527.5
JL2108 (*carB1510*)	37	284.0
	20	271.1

[a] CPSase activity was assayed at 37°C.

CPSase was stored in 0.1 M potassium phosphate (pH 7.6) at 4°C. Although filter-sterilized CPSase solutions are stable for several months under these conditions, all kinetic and calorimetric experiments were performed within a few days of preparation of the enzyme to avoid possible changes during storage.

Differential scanning calorimetry. Calorimetric scans were performed with an MC-2 differential scanning calorimeter equipped with a DA-2 data acquisition and analysis system (Microcal Inc., Amherst, Mass.). All calorimetric experiments were performed with protein dissolved in 0.1 M potassium phosphate (pH 7.6) containing 1.0 mM EDTA. Preparations were equilibrated with the buffer (and ligands, if present) by extensive dialysis. Protein concentrations were determined by the Bradford method (8). Scans were initially run at a rate of 60°C h^{-1}. Under these conditions, an exotherm was observed following the denaturational endotherm. When the scanning rate was reduced to 20°C h^{-1}, the exotherm was eliminated. Similar results have been reported for aspartate carbamoyltransferase (11).

Immunotitration of CPSase. Immunoglobulins were purified as previously described (21) from rabbit antiserum raised against homogeneous CPSase obtained from the wild-type parent grown at 37°C. For immunotitration of CPSase, an appropriate dilution of the immunoglobulin fraction was added in increasing amounts to 100-μl aliquots of CPSase preparation and the final volume was adjusted to 115 μl with 10 mM potassium phosphate, pH 6.8. After incubation for 15 min at 37°C and for 45 min at 4°C, the samples were then microcentrifuged at 12,000 × *g* for 5 min and the supernatants were assayed for CPSase activity.

RESULTS

Kinetic properties of wild-type CPSase depend on growth temperature. Studies on CPSase from a cold-sensitive mutant showed that kinetic and immunological properties of CPSase were dependent on the growth temperature. To assess the significance of this observation, we purified CPSase from a wild-type strain carrying the wild-type *carAB* operon on a multicopy plasmid (19). The plasmid-encoded CPSase was purified to homogeneity from cells grown at 37 and 20°C. The two preparations were characterized at 37°C with respect to their kinetic properties as well as to their sensitivity to the allosteric inhibitor UMP. The results are

TABLE 2. Kinetic parameters of homogeneous wild-type CPSase preparations obtained from cells grown at 37 and 20°C

Parameter[a]	Growth temp	
	37°C	20°C
K_m for glutamine (mM) at 12 mM MgATP	0.28	0.21
K_m for ammonium chloride (mM) at 1.4 mM MgATP	55	132
$S_{0.5}$ for MgATP (mM) at 10 mM glutamine	1.3	1.5
$I_{0.5}$ for UMP (μM) at 1.4 mM MgATP and 10 mM glutamine	22	18

[a] CPSase was assayed at 37°C.

shown in Table 2. The K_m for glutamine, $S_{0.5}$ (substrate concentration that yields one-half the maximal velocity) for MgATP, and $I_{0.5}$ (concentration required for half-maximal inhibition) for UMP of the plasmid-encoded CPSase synthesized at 37°C are very similar to those previously reported for the chromosomal-encoded CPSase from *S. typhimurium* (3). Comparison of the kinetic parameters for the plasmid-encoded CPSases synthesized at 37 and 20°C shows that the K_m for ammonium chloride for the enzyme synthesized at 20°C is significantly higher than that for the enzyme synthesized at 37°C (Table 2). These results prompted the examination of CPSase synthesized by *S. typhimurium* at a temperature closer to the minimal temperature for growth. Accordingly, CPSase was purified to homogeneity from cells grown at 15°C. The specific activity of CPSase synthesized at 15°C by wild-type *S. typhimurium* was 60% of that synthesized at 37°C (Table 1). Kinetic analysis showed that CPSase synthesized at 15°C differed in the K_m for ammonia (Fig. 1) as well as in the K_m and V_{max} for glutamine (Fig. 2) from the enzyme synthesized at 37°C.

The dependence of the kinetic parameters for CPSase on the growth temperature is clearly shown by the values of the ratio of glutamine- to ammonia-dependent activities of CPSase preparations. This ratio is 1.9, 3.9, and 4.7 for CPSase synthesized at 37, 20, and 15°C, respectively.

The kinetic parameters indicated above were confirmed with two independent purifications for each growth temperature. The reproducibility of these parameters precludes the possibility that the observed differences resulted from an artifact during purification. Rather, different CPSase conformations are produced at different growth temperatures by *S. typhimurium*. These conformations are stable such that they are unaffected by the changes in temperature and ionic strength encountered during purification and assay.

Wild-type CPSase synthesized at 15°C is less thermostable than that synthesized at 37°C. The thermal denaturation of CPSase was examined by differential scanning calorimetry. A plot of excess heat capacity against temperature is shown in Fig. 3a for CPSase produced by *S. typhimurium* at 37°C and scanned at a rate of 20°C h^{-1}. The curve is characterized by a major peak with a temperature of maximum excess heat capacity (T_m) of 58.15°C. A minor peak (peak I of Fig. 3a) with a T_m of 42.05°C is also evident. This minor endotherm reflects the dissociation of dimeric [$(\alpha\beta)_2$] and tetrameric [$(\alpha\beta)_4$] species of CPSase shown (27) to be in equilibrium with the monomeric species ($\alpha\beta$) in the presence of phosphate. This conclusion is based on two findings. The minor endotherm was absent in buffers that do not permit self-association of CPSase (data not shown); it was enhanced in the presence of ornithine (Fig. 3b) or UMP (Fig. 3c), which promote the conversion of CPSase to dimer and tetramer, respectively (6, 27). The T_m for the major peak (peak II of Fig. 3) was independent of CPSase concentration, indicating that the two subunits of the species do not dissociate during denaturation (32). At the completion of the scans, the proteins had precipitated and no denaturational endotherms were observed on rescanning, indicating that the thermal

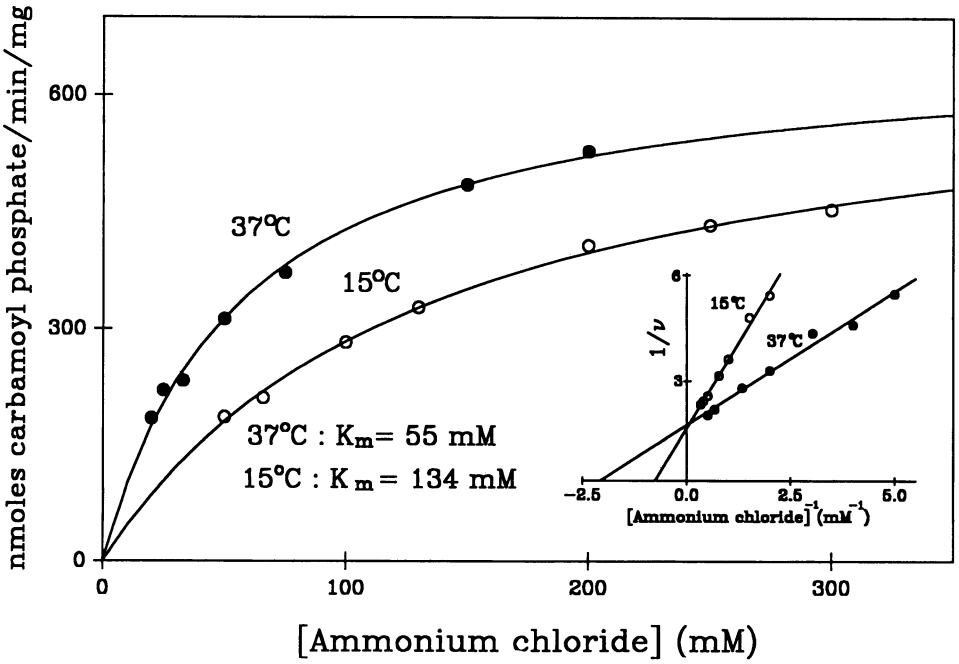

FIG. 1. Ammonium chloride saturation curves for CPSase from wild-type cells grown at 37 and 15°C. Inset shows double-reciprocal plots of the data. The enzyme was assayed at 37°C as described in Materials and Methods except that the concentration of ammonium chloride was varied as indicated.

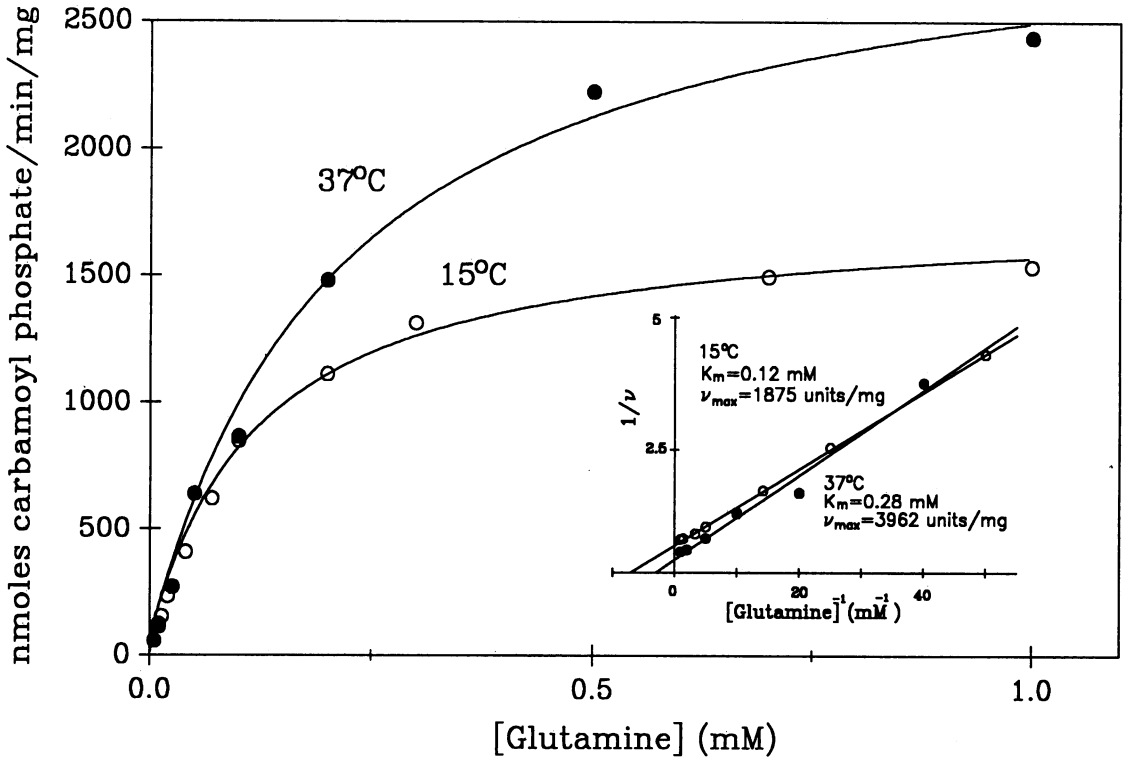

FIG. 2. Glutamine saturation curves for CPSase from wild-type cells grown at 37 and 15°C. Inset shows double-reciprocal plots of the data. The enzyme was assayed at 37°C as described in Materials and Methods except that the concentration of glutamine was varied as indicated.

denaturation of CPSase was irreversible under the conditions employed.

T_m values for oligomer $[(\alpha\beta)_n]$ dissociation and denaturation of CPSase monomer ($\alpha\beta$) are shown in Table 3. Although the T_m value for oligomer dissociation is enhanced by ornithine or UMP, neither allosteric effector influences the T_m value for the denaturational endotherm. Significantly, the T_m values for the denaturational endotherm of CPSase synthesized at 15°C were lower by 2.1 to 2.2°C than those for the enzyme synthesized at 37°C.

The denaturational T_m is a reliable parameter that can be determined accurately (±0.05°C). The difference in T_m values between CPSases synthesized at 37 and 15°C was confirmed with two independently purified CPSase preparations. The difference in stability (ΔG) of the conformations synthesized at 37 and 15°C can be estimated from the difference in T_m values to be 4,500 cal/mol (9).

CPSases synthesized at 37 and 20°C by a cold-sensitive mutant differ significantly in their kinetic properties. CPSase from strain JL2108 grown at 37 and 20°C in glucose minimal medium was purified to homogeneity (see Materials and Methods). Because the strain is an auxotroph at low temperature, the 20°C culture was initially grown at 37°C in the presence of limiting concentrations of arginine (5 μg/ml) and uracil (2.5 μg/ml) and shifted to 20°C at an optical density at 420 nm of 0.3. After the shift to 20°C, the specific growth rate progressively declined and CPSase became derepressed from a level of 0.92 to 11.41 nmol/min per mg of protein. The culture was harvested at an optical density at 420 nm of 0.6.

Detailed analysis revealed that CPSase synthesized at 20°C differed in kinetic properties from that synthesized at 37°C. The most striking difference was observed in the glutamine saturation curves (Fig. 4). The double-reciprocal plot for glutamine with CPSase synthesized at 37°C was linear (Fig. 4A), yielding a K_m of 1.8 mM. In contrast, the double-reciprocal plot for CPSase synthesized at 20°C was not linear (Fig. 4A). The latter can be interpreted on the basis of a single CPSase form that exhibits negative cooperativity, an interpretation supported by a Hill plot of the data. The Hill plot (Fig. 4B) is linear ($r^2 = 0.998$) and yields an $S_{0.5}$ for glutamine of 6.8 mM and a Hill coefficient of 0.8. Studies with other enzymes (12, 29) showed that an enzyme with a single binding site for a substrate, as is the case with binding of glutamine to CPSase, can exhibit cooperativity.

The glutamine saturation curves were also determined at an assay temperature of 20°C (data not shown). The resulting $S_{0.5}$ and Hill coefficient values are compared with those obtained from Fig. 4 (assayed at 37°C) in Table 4. Values for $S_{0.5}$ for glutamine (obtained from Hill plots of the data) were lower when the assays were performed at 20°C. However, $S_{0.5}$ values for CPSase synthesized at 20°C were severalfold higher than those for CPSase synthesized at 37°C, regardless of the assay temperature. Accordingly, if the reduced affinity for glutamine is the basis for the inability of the cold-sensitive mutant to grow at 20°C, this inability reflects the effect of the synthesis temperature on the properties of the enzyme rather than a direct effect of temperature on the folded enzyme.

T_m for CPSase from cold-sensitive mutant is significantly lower than that for wild-type enzyme. Differential scanning calorimetry scans for mutant CPSase (data not shown) showed a single endotherm with a T_m of 47.45°C at a scanning rate of 60°C h^{-1}. The absence of a minor endotherm reflecting oligomer dissociation is likely the result of this dissociation being combined with the denaturational endotherm, which has a T_m lower than that for wild-type

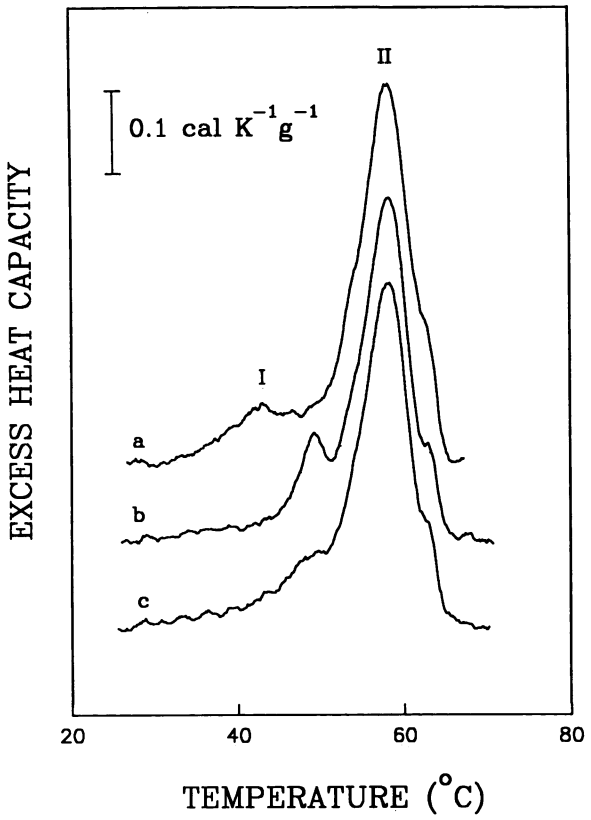

FIG. 3. Plot of excess heat capacity against temperature for CPSase from wild-type cells grown at 37°C. The scan rate was 20°C h^{-1}. Endotherm I reflects dissociation of oligomeric CPSase species $[(\alpha\beta)_n]$. Endotherm II reflects denaturation of the monomeric species ($\alpha\beta$). All experiments were performed in 0.1 M potassium phosphate (pH 7.6) containing 1.0 mM EDTA. a, No ligands at a protein concentration of 1.7 mg/ml; b, plus 5 mM ornithine at a protein concentration of 2.0 mg/ml; c, plus 0.1 mM UMP at a protein concentration of 2.1 mg/ml.

CPSase. This value is 14°C less than that for the parent CPSase when scanned at the same rate. This significant difference in stability indicates that the mutation likely affects a residue that is normally buried in the native conformation. Studies with other proteins showed that substitutions of solvent-exposed residues have little effect on thermal stability (5).

The T_m value for CPSase from the cold-sensitive mutant synthesized at 20°C was identical to that for CPSase synthesized at 37°C. However, analysis of differential scanning calorimetry scans for CPSase preparations from the cold-sensitive mutant indicated that the denaturational enthalpy

TABLE 3. Effect of growth temperature of *S. typhimurium* on T_m values for cloned wild-type CPSase

Growth temp (°C)	Ligand	T_m (°C)	
		Oligomer $[(\alpha\beta)_n]$ dissociation	Denaturational endotherm
37	None	42.05	58.15
	Ornithine	48.97	58.05
	UMP	48.64	58.15
15	None	39.14	55.95
	Ornithine	45.78	55.95

(ΔH_{cal}) for CPSase synthesized at 20°C (458 kcal mol^{-1}) is lower than that for the enzyme synthesized at 37°C (729 kcal mol^{-1}).

CPSase synthesized at 20°C by the cold-sensitive mutant differs in sensitivity to immunoglobulins from that synthesized at 37°C. Immunoglobulin fraction raised against homogeneous wild-type CPSase was used in enzyme inactivation experiments with CPSases synthesized by the wild-type and cold-sensitive strains. The results (Fig. 5) show that CPSases synthesized at 37 and 20°C by the wild-type parent exhibit only minor differences in their sensitivities in immunotitration. In contrast, CPSases produced at the two temperatures by the cold-sensitive mutant exhibited significant differences in inactivation experiments. The conformation produced at 37°C by the cold-sensitive derivative was initially stimulated by the addition of immunoglobulin and was inactivated to a lesser degree than the conformation produced at 20°C (Fig. 5). Immunological studies with mutant β-galactosidases (10) led to the proposal that antibodies can activate mutant enzymes by inducing changes to nativelike conformations.

DISCUSSION

The results presented in this report establish that the growth temperature of wild-type *S. typhimurium* influences the thermal stability as well as certain kinetic properties of CPSase. Synthesis of more thermostable enzymes at higher growth temperatures by thermophiles has been reported (22), and differential expression of duplicate genes was proposed to account for the observed differences (35). In the case of the *carAB* operon of *S. typhimurium*, the possibility of duplicate genes is precluded. All available mutants including cold-sensitive mutants map in the same *carAB* locus (1, 37). This operon has been cloned and fully characterized (19, 23). Further, there is no evidence for chemical modification of CPSases from enteric bacteria. Accordingly, the effect of growth temperature on properties of CPSase can be accounted for only by an effect on the folding pathway.

The ratio of the rates of glutamine- and ammonia-dependent activities of CPSase is a sensitive index of this variation. The ammonia-dependent rate is a function of the large subunit alone, whereas the glutamine-dependent rate is a function of the holoenzyme (3). Previous work (3, 16, 30) provided evidence for strong interactions between the large and small subunits, and the ratio obtained with the two substrates must depend on the conformation of the holoenzyme. The value of this ratio for the form of CPSase synthesized at 15°C is 2.5-fold higher than for the form synthesized at 37°C. These results indicate that different stable conformations of wild-type CPSase are synthesized at the two temperatures.

The synthesis of different stable conformations of CPSase is supported by the results of calorimetric studies. The temperature of maximum excess heat capacity (T_m) for CPSase synthesized at 15°C was 2°C lower than that synthesized at 37°C. This difference in T_m values corresponds to a difference in stability (ΔG) of 4,500 cal/mol, which can be attributed to the tertiary and quaternary structure of the monomeric ($\alpha\beta$) species since the dimeric $[(\alpha\beta)_2]$ and tetrameric $[(\alpha\beta)_4]$ species dissociate before the denaturational endotherms. Further, studies on the thermal denaturation of proteins (25) indicate that thermal transitions reflect destruction of quaternary rather than secondary structure. Analysis of the available calorimetric data to be reported elsewhere (unpublished data) shows that the denaturational enthalpy of CPSase synthesized at 15°C is also lower than that for the enzyme synthesized at 37°C.

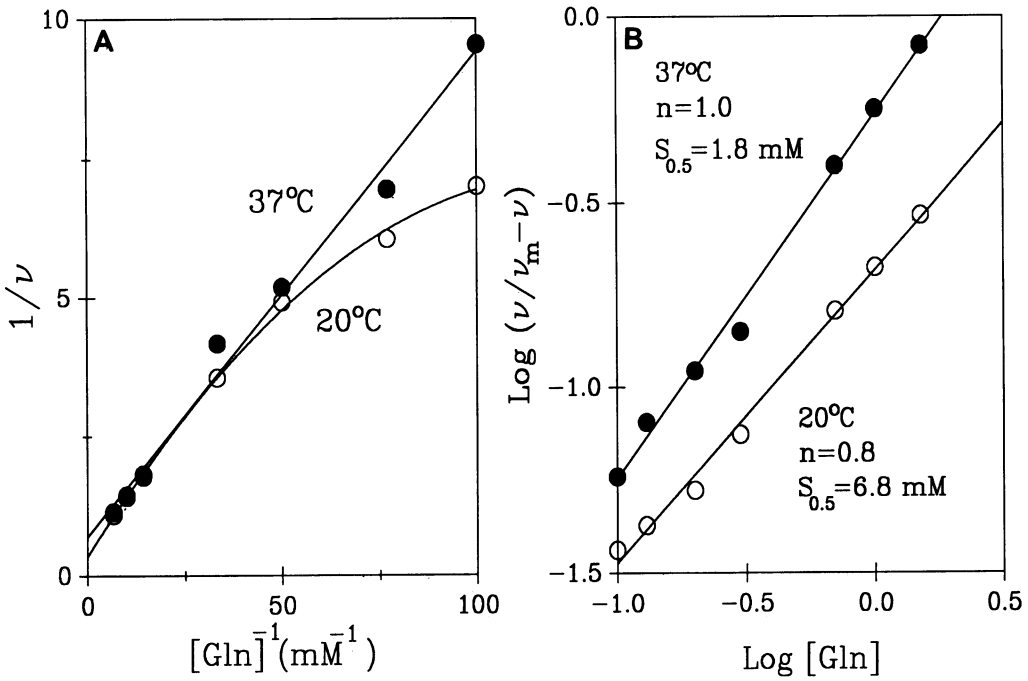

FIG. 4. (A) Double-reciprocal plots for glutamine (Gln) with CPSase from a cold-sensitive mutant (*carB1510*) grown at 37 and 20°C. (B) Hill plots of the data; $S_{0.5}$ and n (Hill coefficient) were calculated by nonlinear regression analysis. The enzyme was assayed as described in Materials and Methods except that ATP and $MgCl_2$ concentrations were 2 and 6 mM, respectively. The concentration of glutamine was varied as indicated.

Numerous authors (14, 15, 18, 36) have questioned the long-held view that protein folding is determined exclusively by thermodynamic factors, i.e., that the native protein is the most stable conformation. It is increasingly recognized that kinetic factors are important determinants of choices among folding pathways: the native structure of a protein is not necessarily the structure with the lowest Gibbs free energy; rather, it is the lowest free energy structure that is kinetically accessible (36). The hypothesis of kinetic control of protein folding proposes that folding occurs through a unique pathway leading to a metastable state (13).

Our current hypothesis holds that the folding pathway of CPSase proceeds from domain folding to subunit folding to assembly of the two subunits. We further assume that additional conformational changes follow subunit assembly, thus completing the folding process. These assumptions are based on two concepts. The concept of independent folding of domains comes from studies on protein fragments (28, 36) including those of rat liver CPSase (26). The concept of postassembly folding comes from observed conformational changes of assembled oligomeric proteins (13, 34). This last step is essential for expression of full catalytic and allosteric properties of the enzyme (13).

It should be stressed that interdomain as well as intersubunit contacts are mainly hydrophobic (13). On the basis of solvent transfer experiments using a hydrocarbon model, Baldwin (7) concluded that the major contribution to enthalpy change of protein unfolding at high temperatures comes from hydrophobic interactions. Hydrophobic interactions are weaker at lower temperatures (7). Accordingly, the effect of temperature during synthesis on the choice of the folding pathway followed by wild-type CPSase can be interpreted to be directed by weakening of hydrophobic interactions either between domains or between subunits. The consequence is synthesis of different conformations at 37 and 15°C.

The results reported here establish that the cold-sensitive phenotype results from cold-sensitive synthesis rather than cold lability. In fact, CPSase titer is derepressed at 20°C but the altered properties of the enzyme synthesized at this temperature render it physiologically nonfunctional. The observed differences in kinetic, immunological, and calorimetric properties of the two forms synthesized by the mutant at 37 and 20°C support the hypothesis that mutations conferring cold sensitivity identify amino acid residues that are critical in the folding of CPSase.

Studies by King and co-workers (20) indicate that all amino acid residues in a polypeptide sequence do not contribute equally to the folding pathway; rather, specialized sequences may direct the folding pathway. These authors used temperature-sensitive synthesis mutants to characterize such specialized sequences for the tail spike protein of phage P22 of *S. typhimurium*. It will be of interest to compare the nature and organization of amino acid residues

TABLE 4. Effect of assay temperature on parameters[a] of glutamine saturation curves for CPSases synthesized at 37 and 20°C by the cold-sensitive mutant

Growth temp (°C)	Assay at 37°C		Assay at 20°C	
	$S_{0.5}$ (mM)	n_H	$S_{0.5}$ (mM)	n_H
37	1.8	1.0	0.40	1.3
20	6.8	0.8	2.17	0.7

[a] Values for $S_{0.5}$ (substrate concentration that yields one-half the maximal velocity) and n_H (Hill coefficient) were determined from Hill plots. CPSase was assayed as described in Materials and Methods except that ATP and $MgCl_2$ concentrations were 2 and 6 mM, respectively. Glutamine was varied as shown in Fig. 4.

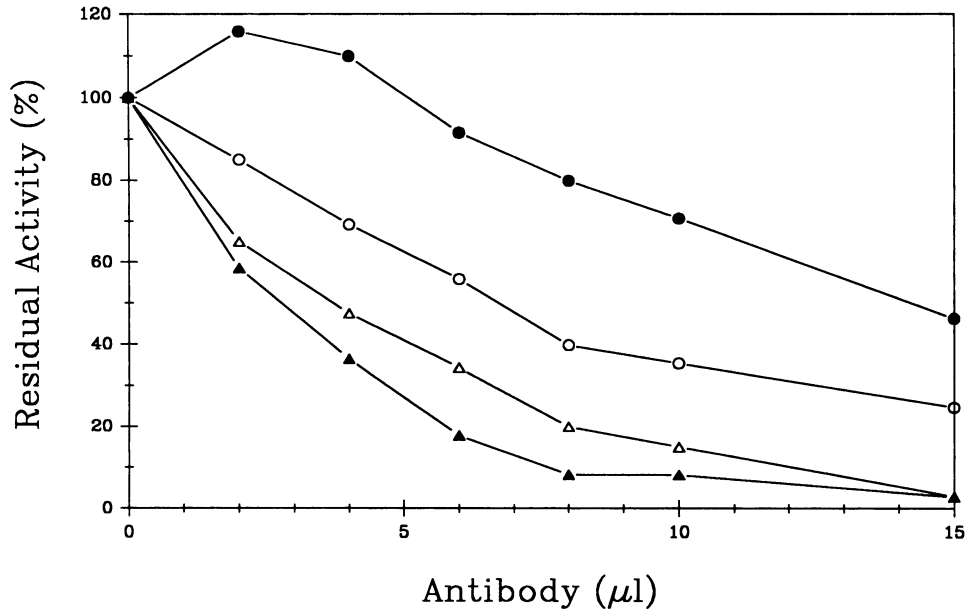

FIG. 5. Inactivation of CPSase activity by immunoglobulins raised against homogeneous wild-type CPSase from cells grown at 37°C. Symbols; ▲, wild-type cells grown at 37°C; △, wild-type cells grown at 20°C; ●, cold-sensitive mutant grown at 37°C; ○, cold-sensitive mutant grown at 20°C.

identified by cold-sensitive synthesis mutants of *carAB* with those identified by King and co-workers (20).

Previous studies on cold-sensitive mutations led to the conclusion (17) that in contrast to heat-sensitive mutations, mutations conferring cold sensitivity are restricted to a limited number of genes. Most of the mutations resulting in cold sensitivity are in genes encoding allosteric enzymes or ribosomal proteins (17). The function of both types of proteins is expected to depend on hydrophobic interactions that are weakened at low temperature. The results reported here show that the effect of temperature on folding of CPSase is evident in wild-type *S. typhimurium*. As the growth temperature approaches the minimal temperature for growth of *S. typhimurium*, the conformation of CPSase may be increasingly altered. Accordingly, it is reasonable to propose that the basis of minimal temperature for growth includes the combined effects of temperature on the folding of a number of allosteric proteins that occupy central positions in metabolic pathways. The results of studies on CPSase from the wild type have shown that an environmental factor (temperature) can change protein folding. In the mutant enzyme, these changes have significant biological consequences.

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