

# Nucleotide ligands protect the inter-domain regions of the multifunctional polypeptide CAD against limited proteolysis, and also stabilize the thermolabile part-reactions of the carbamoyl-phosphate synthase II domains within the CAD polypeptide

Elizabeth A. CARREY

Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

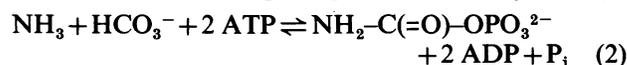
Improved methodologies are described which allow the measurement of the part-reactions, with glutamine or ammonia as nitrogen donor, of mammalian carbamoyl-phosphate synthase II (EC 6.3.5.5) through the incorporation of [<sup>14</sup>C]bicarbonate into either carbamoyl phosphate or carbamoylaspartate. The enzyme is part of the multifunctional polypeptide (CAD) which also comprises the pyrimidine-biosynthetic enzymes aspartate transcarbamoylase (EC 2.1.3.2) and dihydro-ototase (EC 3.5.2.3). The conformational stability of the carbamoyl-phosphate synthase was investigated through the inactivation of the part-reactions which occurred during incubation at 37 °C. The domain involved in the removal of the amide N from glutamine was more thermolabile than the ammonia-dependent synthase moiety. The former activity was stabilized in the presence of sodium aspartate or MgATP, whereas the latter was stabilized by MgATP and MgUTP. Binding of MgUTP and MgATP to CAD restricted the initial proteolysis by trypsin and elastase of one or both regions linking the carbamoyl-phosphate synthase domain to the other major domains. A model is described to account for both aspects of nucleotide binding to CAD; these stabilizing effects may be important in the cell, where similar concentrations of nucleotides are found.

## INTRODUCTION

The multifunctional polypeptide CAD, found in the cytoplasm of mammalian cells [1], contains the first three activities in pyrimidine biosynthesis, i.e. the glutamine-dependent carbamoyl-phosphate synthase (CPSaseII), aspartate transcarbamoylase (ATCase) and dihydro-ototase (DHOase). Bifunctional polypeptides containing the first two enzyme activities are found in *Neurospora* [2] and yeast [3]. In common with other multifunctional proteins, CAD comprises a number of compact globular domains connected by more exposed polypeptide regions, which are vulnerable to proteolytic enzymes [4]. Controlled proteolysis with low concentrations of elastase [5] and trypsin [6] has demonstrated the multidomain nature of CAD, and has generated active fragments corresponding to the ATCase domain, of approx. 40 kDa [7], and the DHOase domain, of approx. 43 kDa [8].

The CPSaseII activity comprises a complex of domains accounting for a large moiety of approx. 150 kDa in the centre of the CAD polypeptide. It has been proposed that the part-reactions, (1) and (2) below, and the binding of the allosteric effectors involve separate domains within this complex [8]. The glutamine-dependent activity can be divided into a glutaminase reaction (1) and the transfer of the ammonia group to the active site of the

carbamoyl-phosphate synthase, followed by the reaction (2) common to all CPSase isoenzymes:



In CPSaseII the synthesis of carbamoyl phosphate by reaction (2) is regulated by the nucleotides *PPRibP* (positive effector) and UTP, the negative effector and end-product of the pathway (E. A. Carrey, unpublished work), whereas other CPSases require *N*-acetylglutamate as a positive effector [9]. In prokaryotes, glutaminase forms a smaller subunit associating non-covalently with the CPSase; the sequence of the CPSaseI from rat mitochondria demonstrates that these mammalian enzymes have evolved through a fusion between the glutaminase and synthase genes [10, 11]. It is possible that the same fusion is ancestral to the CPSaseII enzyme, which has an integral, functional, glutaminase. Despite the differences in specificity for allosteric effector molecules, the CPSase enzymes exhibit immunological cross-reactivity [9], suggesting that the synthase and glutaminase domains make up a large part of the common conformation which is recognized by antibodies.

If the CPSase enzymes have similar conformations, they may also exhibit similar resistance to denaturing

Abbreviations used: CAD, the multifunctional polypeptide containing the activities ATCase, CPSaseII and DHOase; ATCase, aspartate transcarbamoylase (EC 2.1.3.2); CPSaseI, ammonia- and *N*-acetylglutamate-dependent carbamoyl-phosphate synthase (EC 6.3.4.16); CPSaseII, glutamine-dependent carbamoyl-phosphate synthase (EC 6.3.5.5); DHOase, dihydro-ototase (EC 3.5.2.3); *PPRibP*, 5-phosphoribosyl 1-pyrophosphate; p[CH<sub>2</sub>]<sub>ppA</sub>, adenosine 5'-[β,γ-methylene]triphosphate.

influences, or a similar pattern of vulnerable polypeptide regions attacked by limited proteolysis between the component domains. The unfolding process in a number of proteins has been investigated through the susceptibility of exposed or flexible regions to proteolysis [12]. Since metabolic channelling in enzyme complexes such as CAD may arise from the close proximity of enzyme active sites, this implies that changes in conformation on binding allosteric effectors or active-site-directed ligands may be transmitted to a neighbouring domain, even one catalysing a distinct reaction, and may alter the range of proteolytic products. In this respect, interesting preliminary work has shown [8] that the digestion by elastase of the mitochondrial CPSaseI (subunit size 165 kDa) in the presence of MgATP generates different polypeptide products compared with digestion in the absence of ligands. In particular, an enzymically active fragment of molecular mass 125 kDa, which may represent the minimal combination of substrate-binding domains, is liberated from the CPSaseI-ATP complex. Proteolysis of CAD by low concentrations of elastase in the absence of ligands generated a polypeptide of approx. 150 kDa, which corresponded to the central CPSaseII moiety, but the enzyme activity of fragments below 190 kDa in size was reported to be very labile [5], in contrast with the CPSaseI fragment.

The work described in the present paper extends a previous observation [13] that both MgATP and MgUTP retarded the early steps in controlled proteolysis of CAD by trypsin and elastase at 37 °C. This may have been effected through the formation of a CPSase-ligand complex with a more stable conformation and less exposed inter-domain regions than the unliganded form. The possibility of denaturation within the domains during incubation at 37 °C was investigated through the enzyme activities of the central domain complex. The glutaminase part-reaction and the ammonia-dependent synthase were studied separately by providing either glutamine or  $\text{NH}_4\text{Cl}$  as the ammonia donor for carbamoyl phosphate synthesis.

## EXPERIMENTAL

### Materials

Sterile plastic flasks and tubes for cell culture were obtained from Flow Laboratories and Sterilin respectively; Dulbecco's modified Eagle's medium, foetal-calf serum, antibiotics and trypsin suspension were from Flow Laboratories. Blue G and Blue R stains were obtained from Serva (Heidelberg, Germany).  $\text{NaH}^{14}\text{CO}_3$  (>1.85 GBq/mmol) was obtained from Amersham; Optiphase multipurpose scintillant was from Fisons; ATP,  $\text{p}[\text{CH}_2]\text{ppA}$ , pig elastase suspension, bovine trypsin and phenylmethanesulphonyl fluoride were from Boehringer Mannheim. The acrylamide used in electrophoresis was BDH Electran grade 1; other reagents for electrophoresis were obtained from Bio-Rad. Tris, Hepes and UTP were obtained from Sigma.

### Cell culture and enzyme purification

The transformed hamster kidney cell line 165-28, which overproduces CAD, was generously provided by Dr. George Stark (ICRF, London). Cells were grown in 75  $\text{cm}^2$  polystyrene culture flasks in Dulbecco's modified Eagle's medium containing 20 mM-Hepes, with the addition of 10 mM- $\text{NaHCO}_3$ , 4 mM-glutamine, 2% (v/v)

penicillin/streptomycin solution and 10% (v/v) foetal-calf serum. The Hepes buffer eliminates the need for gaseous  $\text{CO}_2$  to maintain the pH of the solution. Cells were harvested by using a 0.025% solution of trypsin in minimal medium, and were then suspended in medium supplemented as described above, except that benzamidine (final concn. 2.5 mM) replaced the foetal-calf serum.

The purification procedure of Coleman *et al.* [14] was followed, with some modifications; after the differential centrifugation and the removal of nucleic acids, the protein was precipitated with 0.65 vol. of saturated  $(\text{NH}_4)_2\text{SO}_4$  in 50 mM-Hepes (adjusted to pH 7.4 with KOH)/10% (w/v) glycerol/1 mM-EDTA. The pellet was resuspended in a minimal volume of 0.1 M-Hepes/KOH (pH 7.0)/5% glycerol/30% (v/v) dimethyl sulphoxide/1 mM-dithiothreitol. This preparation, usually 10 mg of protein/ml, was stable and enzymically active for several months when stored at -70 °C. H.p.l.c. gel filtration in the TSK G4000 SW (Anachem) column with the buffer in which the  $(\text{NH}_4)_2\text{SO}_4$  pellet was resuspended removed some contaminating proteins, with little increase in specific enzyme activity. The purity of the preparation was demonstrated in Blue R-stained electrophoresis gels as illustrated in Figs. 4 and 5; densitometric scanning of the control lanes indicated that the CAD band made up more than 95% of the stained intensity.

### Protein assay

Protein concentration was measured by the Bradford [15] method, with 30 mg of Blue G/litre of reagent. The colour reaction with CAD was measured immediately after reagent had been added to the CAD sample, because fading had been noticed when CAD (but not the standard, bovine serum albumin) was used. Values obtained in this way were within 5% of the protein concentration obtained through refractometry in the Spinco model E analytical ultracentrifuge [16]. The absorbance index,  $A_{280}^{1\%}$ , of purified CAD was 13.0.

### Enzyme assays

Carbamoyl-phosphate synthase was assayed by modifications of previously published methods [17, 18]. A total incubation volume of 0.25 ml contained 0.1 M-Tris adjusted at room temperature with HCl to correspond to pH 7.5 at 37 °C, 0.1 M-KCl, 7.5% dimethyl sulphoxide, 2.5% glycerol, 25 mM- $\text{MgCl}_2$ , 15 mM-ATP (neutralized with KOH), 1 mM-dithiothreitol, and other components as specified below. The reaction was initiated by adding 8  $\mu\text{l}$  of CAD solution (0.5–1  $\mu\text{g}$  of protein) and 25  $\mu\text{l}$  of  $\text{NaH}^{14}\text{CO}_3$  (0.2 M; 0.75 Ci/mol), vortex-mixing and returning to incubation at 37 °C. After 15–20 min the assays were stopped as specified below, and the entire contents of the reaction vials were dried, and then resuspended in 0.4 ml of water and 4 ml of scintillant in glass scintillation vials. 'Blank' vials contained all the substrates but no enzyme. Specific radioactivity was measured by adding a portion of  $\text{NaH}^{14}\text{CO}_3$  to scintillation vials which contained dried-down incubation mixture and non-radioactive  $\text{NaHCO}_3$ .

Three assay systems were derived from the published methods, each using as nitrogen donor either glutamine (3–4 mM) or  $\text{NH}_4\text{Cl}$ , pH 7.5 (10–15 mM). In system 1, the substrate mixture was as described above, and the assay was stopped by mixing with 0.15 ml of 2 M- $\text{NH}_4\text{Cl}$  and heating the tubes at 90 °C for 10–20 min. This process

quantitatively converts carbamoyl phosphate into urea [19]. The unchanged  $\text{NaHCO}_3$  was driven off as  $\text{CO}_2$  by adding 0.1 ml of 14% (v/v)  $\text{HClO}_4$ , and the assay mixtures were dried and resuspended for scintillation counting. In assay system 2, the substrate mixture contained sodium aspartate (typically 15 mM), allowing the transcarbamoylase reaction to convert carbamoyl phosphate into carbamoylaspartate. The reaction was stopped with  $\text{NH}_4\text{Cl}$  and acid as in system 1, so the stable products corresponded to both carbamoyl phosphate and carbamoylaspartate. In system 3, the substrate mixture contained sodium aspartate, but the assay was stopped by the addition of 0.25 ml of 7%  $\text{HClO}_4$ . The acid-stable product was carbamoylaspartate only.

**Limited proteolysis**

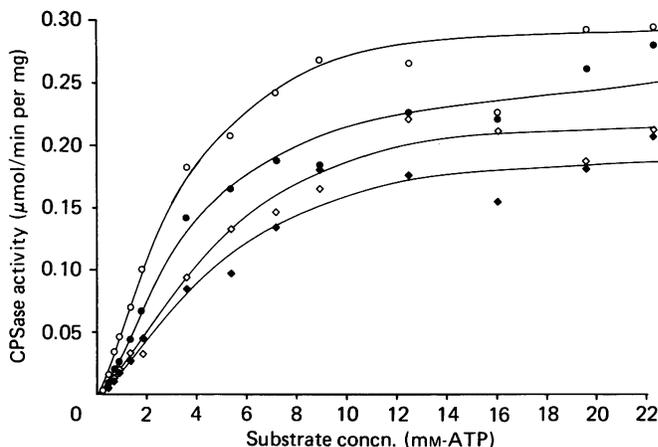
Purified CAD (40  $\mu\text{g}$ ) was incubated in 0.1 M-Hepes/KOH, pH 7.4, containing 4 mM- $\text{MgCl}_2$  and (if required) additional  $\text{MgCl}_2$  to make up twice the concentration of added nucleotide. Nucleotides were used from stock solutions neutralized with KOH and stored at  $-20^\circ\text{C}$ , except for *PPRibP*, which was freshly weighed and used on the same day. After incubation for 10 min at  $37^\circ\text{C}$ , either elastase or trypsin in 30  $\mu\text{l}$  of 0.1 M-Hepes was added to give a total incubation volume of 0.28 ml. The ratios (w/w) of proteinase:CAD were approx. 1:700 for trypsin and 1:140 for elastase. Samples (60  $\mu\text{l}$ ) were removed at timed intervals during the incubations with trypsin or elastase, and were immediately added to 30  $\mu\text{l}$  of Bromophenol Blue electrophoresis sample buffer containing 10% mercaptoethanol, 1 mM-phenylmethanesulphonyl fluoride and 10 mM-benzamidine hydrochloride, and heated at  $90^\circ\text{C}$  for 10 min. An undigested control was incubated for 10 min, added to the Bromophenol Blue solution and heated to  $90^\circ\text{C}$ .

**Electrophoresis**

Samples were applied to 1.5 mm-thick slab gels, comprising a separating gel with a polyacrylamide gradient of 12.5% (bottom) to 5% (top) and a 3% -acrylamide stacking gel [20]. This allowed a good separation of polypeptides within the size range 25–250 kDa. Gels were stained in Serva Blue R (0.6% in 50% methanol/10% acetic acid).

**Computer fitting of kinetic data**

Kinetic parameters were estimated by fitting experimental values to the Hill equation  $v/V_{\text{max}} = s^h / (K + s^h)$ ,



**Fig. 1. Thermal inactivation of glutamine-dependent CPSaseII activity under assay conditions**

Purified CAD (4  $\mu\text{g}/\text{ml}$ ) was incubated at  $37^\circ\text{C}$  in assay buffer containing 3.3 mM-glutamine, 15 mM-aspartate and various concentrations of ATP. The assays were initiated after the indicated intervals by the addition of  $\text{NaH}^{14}\text{CO}_3$  and quenched after 15 min (assay system 3). Preincubation periods: ○, immediate assay; ●, 20 min; ◇, 40 min; ◆, 60 min.

where  $v$  is the apparent initial velocity,  $s$  is the substrate concentration,  $K$  is the apparent  $K_m$ , and  $h$  is the Hill coefficient.

**RESULTS**

**Use of three different systems to measure CPSaseII activity**

The three assay systems described in the Experimental section were developed in order to measure the carbamoyl phosphate production when the ATCase activity was either absent or inhibited. When the ATCase activity was present, any carbamoyl phosphate was channelled to the production of carbamoylaspartate, unless aspartate was omitted from the reaction mixture. The rate of inactivation during incubation at  $37^\circ\text{C}$  was the same for each assay system, confirming that the rate of carbamoyl phosphate synthesis was measured, whether the  $^{14}\text{C}$ -labelled product was carbamoyl phosphate or carbamoylaspartate. Activities measured with

**Table 1. Kinetic parameters of glutamine-dependent carbamoyl-phosphate synthase**

The enzyme was assayed after incubation with a range of ATP concentrations in the assay buffer (containing 15 mM-sodium aspartate and 3.3 mM-glutamine) at  $37^\circ\text{C}$  as described in the legend to Fig. 1. Data, as shown in Fig. 1, were fitted by computer to the Hill equation as described in the Experimental section. The maximum specific activity and apparent  $K_m$  for ATP are shown as means  $\pm$  S.D. The  $s_{0.5}$  is the concentration of ATP giving half-maximal velocity, calculated as the  $h$ th root of  $K_m$ , where  $h$  is the Hill coefficient.

Incubation time before assay (min)	$K_m$ (mM-ATP)	Maximum specific activity ( $\mu\text{mol}/\text{min per mg}$ )	$s_{0.5}$ (mM-ATP)	$h$
0	$4.56 \pm 0.78$	$0.286 \pm 0.009$	2.6	1.6
20	$7.29 \pm 0.98$	$0.296 \pm 0.012$	4.8	1.3
40	$11.60 \pm 1.9$	$0.229 \pm 0.009$	4.5	1.6
60	$10.5 \pm 2.1$	$0.211 \pm 0.012$	4.8	1.5

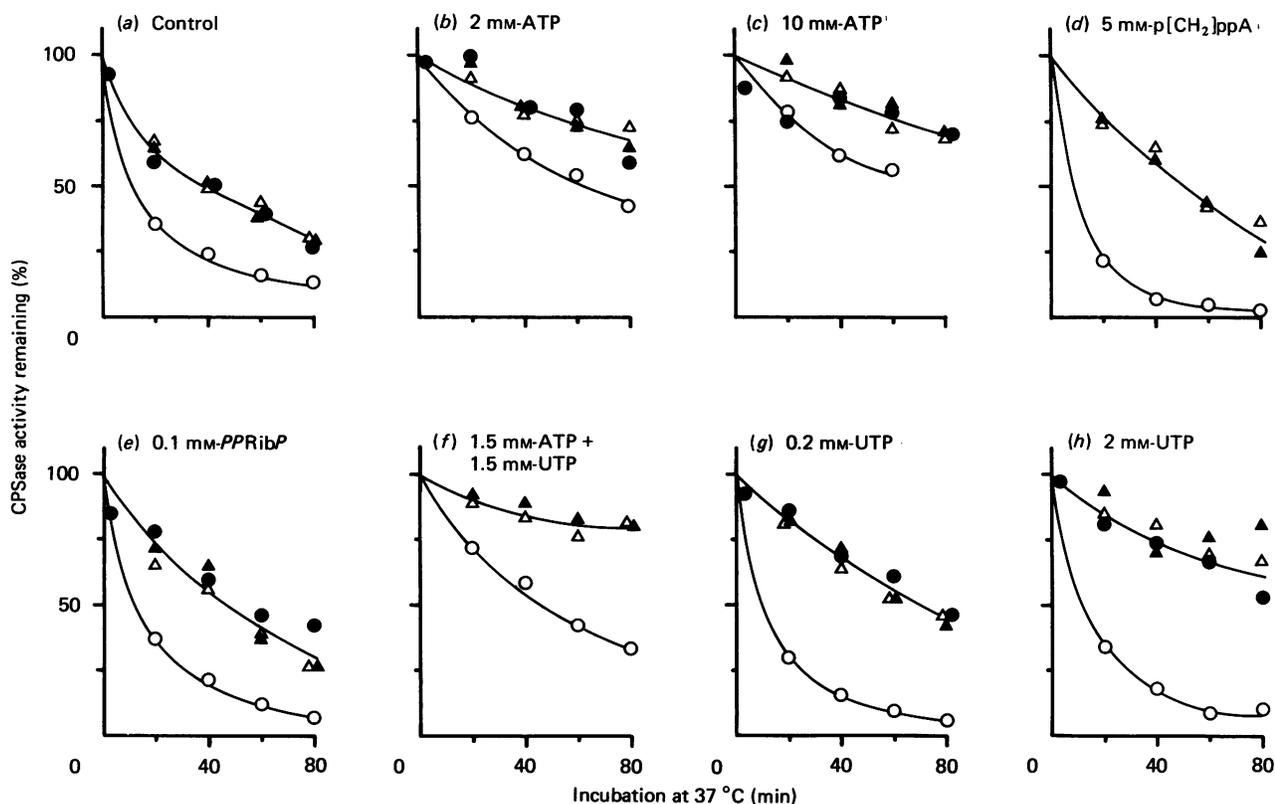


Fig. 2. Thermal inactivation of CPSaseII; remaining activity measured after preincubation at 37 °C

Purified CAD (75  $\mu\text{g}/\text{ml}$ ) was incubated at 37 °C in 0.1 M-Tris/HCl, pH 7.5, containing 0.1 M-KCl, 7.5% dimethyl sulphoxide, 2.5% glycerol, 4 mM-MgCl<sub>2</sub>, 1 mM dithiothreitol, and nucleotide solutions (neutralized with KOH) as indicated, plus additional MgCl<sub>2</sub> to make its concentration twice the nucleotide concentration. At the indicated times, samples (approx. 0.6  $\mu\text{g}$ ) of CAD were removed from the incubations and assayed as follows: ○, 4 mM-glutamine substrate, assay system 1; △, 10 mM-NH<sub>4</sub>Cl substrate, assay system 1; ▲, 10 mM-NH<sub>4</sub>Cl substrate, assay system 3. In a similar experiment, CAD was incubated in the same buffer, containing 25 mM-MgCl<sub>2</sub>, 3.3 mM-glutamine, 15 mM-aspartate, and nucleotides as indicated, and then assayed as follows: ●, 3 mM-glutamine substrate, assay system 3. Points represent data from two or more separate assays. Nucleotide additions were: (a) none; (b) 2 mM-ATP; (c) 10 mM-ATP; (d) 5 mM-p[CH<sub>2</sub>]ppA; (e) 100  $\mu\text{M}$ -PPRibP; (f) 1.5 mM-UTP + 1.5 mM-ATP; (g) 0.2 mM-UTP; (h) 2 mM-UTP.

10–15 mM-NH<sub>4</sub>Cl as substrate in controls were similar to the glutamine-dependent activity (E. A. Carrey, unpublished work).

#### Lablity of CPSaseII in assay conditions

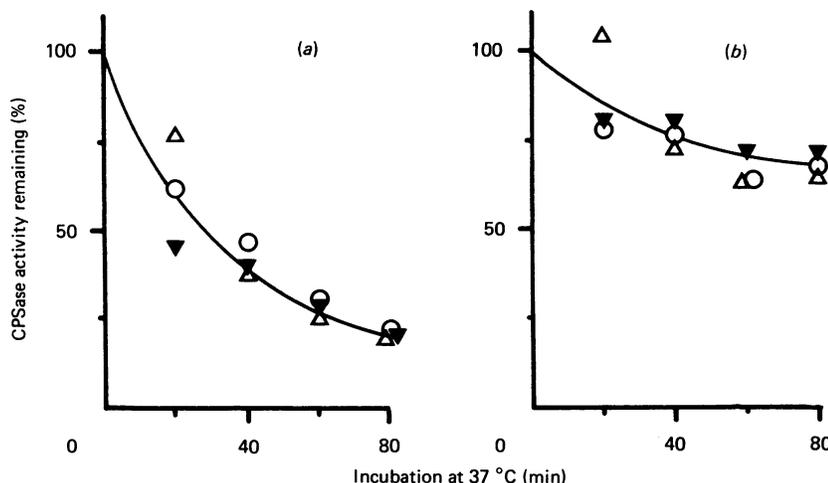
A substantial loss of glutamine-dependent carbamoyl-phosphate synthase activity was observed when purified CAD was equilibrated at 37 °C in assay buffer containing 15 mM-sodium aspartate and a range of ATP concentrations before initiation of the synthase reaction (assay system 3). Fig. 1 shows that the highest enzyme activities were measured when the assay was initiated simultaneously with the addition of CAD. The activity was decreased by about 60% when measured after 1 h equilibration with 1 mM-ATP, and at the highest ATP concentration by about 30%. This lablity of the enzyme under assay conditions implies that assay systems which incorporate an equilibration period at 37 °C [18] before addition of NaH<sup>14</sup>CO<sub>3</sub> will generate substrate (ATP)-saturation curves overlaid by thermal-denaturation effects, causing a lower  $V_{\text{max}}$  and higher  $K_m$  for ATP to be estimated from the substrate saturation curve [21], compared with a simultaneous assay system (see Table 1).

#### Inactivation of CPSaseII by incubation at 37 °C

Fig. 2 illustrates the lablity of the CPSaseII activity when purified CAD protein was incubated at 37 °C before transfer of samples into assays with either NH<sub>4</sub>Cl or glutamine as nitrogen donor.

More than half the glutamine-dependent activity was lost within 20 min during an initial, more rapid, phase of inactivation (Fig. 2a), unless the incubation contained MgATP, which retarded the activity loss at least 4-fold (Figs. 2b and 2c). Neither the allosteric effectors MgUTP or PPRibP nor the substrate analogue p[CH<sub>2</sub>]ppA were able to prevent the decrease in the glutamine-dependent CPSase activity; in fact, the activity remaining after 40 min incubation with UTP (Figs. 2g and 2h) or with 5 mM-p[CH<sub>2</sub>]ppA (Fig. 2d) was appreciably lower than in the control incubation. The inhibitory effects of p[CH<sub>2</sub>]ppA may be entirely due to competition with ATP for the substrate-binding sites, since the evidence from limited proteolysis studies (below) was that p[CH<sub>2</sub>]ppA stabilized the conformation in a similar manner to ATP.

The NH<sub>3</sub>-dependent synthase activity decreased less rapidly in all incubations at 37 °C. A fast initial phase, reaching 50% activity after 40 min, was seen in the



**Fig. 3. Thermal inactivation of glutamine-dependent CPSaseII activity is not affected by added trypsin or proteinase inhibitor**

CAD (80  $\mu\text{g/ml}$ ) was incubated at 37 °C in assay buffer (containing 15 mM-aspartate and 3.3 mM-glutamine) with no ATP (a) or 25 mM-ATP (b). Samples were removed at the indicated times and assayed with 3.3 mM-glutamine as substrate (assay system 3). Additional incubation contents were: ○, none; ▼, trypsin, 80 ng/ml; △, benzamidine hydrochloride, 0.01 M.

control incubation (Fig. 2a), but with 5 mM-p[CH<sub>2</sub>]ppA (Fig. 2d) and 0.1 mM-PPRibP (Fig. 2e) activity was lost monophasically, reaching the same values as the control incubations after 60 min. In contrast with the glutamine-dependent activity, the NH<sub>3</sub>-dependent CPSase was stabilized by either MgATP or MgUTP in the incubation. In the presence of 2 mM-MgATP, 10 mM-MgATP or 2 mM-MgUTP, the NH<sub>3</sub>-dependent activity decreased linearly to about 70% of the initial activity after 80 min at 37 °C. MgUTP at 0.2 mM was nearly as effective as 2 mM-MgUTP in stabilizing the NH<sub>3</sub>-dependent CPSase. The effects of MgUTP and MgATP were not additive in the incubation with 1.5 mM-ATP, 1.5 mM-UTP and 6 mM-MgCl<sub>2</sub> (Fig. 2f), where about 75% of the NH<sub>3</sub>-dependent activity remained after 80 min, as expected for similar concentrations of either nucleotide alone, and MgUTP did not abolish the stabilization of the glutamine-dependent activity induced by MgATP. The ● symbols in Fig. 2 and the data in Fig. 1 demonstrate that the glutamine-dependent activity was more stable when CAD was incubated in assay buffer containing 3.3 mM-glutamine and 15 mM-sodium aspartate. Similar results were obtained with only aspartate in the incubation.

These results are consistent with the view that the part reactions of CPSaseII (i.e. the removal of the ammonia group from glutamine, and the synthase reaction proper) are carried out in discretely folded units or domains which differ in their susceptibility to thermal inactivation. When glutamine was the sole nitrogen donor, the overall synthesis of carbamoyl phosphate was limited by the lower stability of the glutaminase domain at 37 °C. In the assay buffer containing 15 mM-sodium aspartate, the glutaminase domain was at least as stable as the synthase, so that the lability of the NH<sub>3</sub>-dependent reaction became limiting.

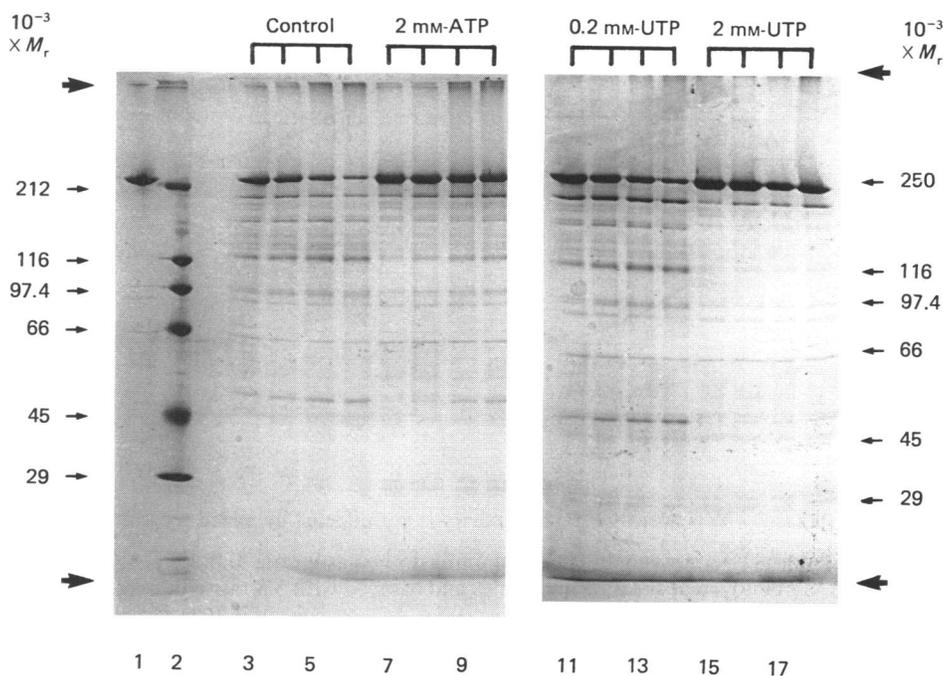
In previous studies of the glutamine-dependent CPSaseII from rat liver, 15 mM-NaHCO<sub>3</sub> provided a small additional stabilization during preincubation with MgATP at 37 °C [21]. No stabilization of either part-reaction was observed with 20 mM-NaHCO<sub>3</sub>

(E. A. Carrey, unpublished work), but, because dissolved CO<sub>2</sub> was not rigorously excluded from the solutions used in the experiments, it is likely that sufficient NaHCO<sub>3</sub> was formed to bind at each CPSase active site. Mori *et al.* [22] found that 0.3 mM-UTP and 50  $\mu\text{M}$ -PPRibP were approximately as effective as 10 mM-ATP in retarding the loss of glutamine-dependent activity of the rat liver enzyme in 10% glycerol at 18 °C. However, this group has reported anomalous behaviour of these allosteric effectors in high concentrations of glycerol or dimethyl sulphoxide [23], which may account for the differences from the behaviour reported here and shown in Fig. 2.

Mally *et al.* [5] reported that the early stages of digestion of CAD by elastase caused a rapid decrease in glutamine-dependent CPSaseII activity. As a control for the experiments described above, a preparation of the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitate of CAD was incubated in assay buffer, with either trypsin [approx. 1:10<sup>3</sup> (w/w) with CAD], or benzamidine, to inhibit any proteinase that might have co-purified with CAD. The fall in the glutamine-dependent CPSase activity (Fig. 3) was the same in each group of incubations, and MgATP had the same stabilizing effect regardless of added trypsin. Analysis by SDS/polyacrylamide-gel electrophoresis (results not shown) demonstrated that CAD had been 'nicked' in the incubations containing trypsin, but not in the control incubations or with benzamidine. Some protection against trypsin was observed in those incubations containing nucleotides. Thus, although additional trypsin at a ratio of 1:10<sup>3</sup> (w/w) caused significant proteolysis of CAD over the period of incubation, no additional loss of CPSaseII activity was caused by tryptic proteolysis. The loss of NH<sub>3</sub>- or glutamine-dependent activity is therefore not a direct result of cleavage, but reflects conformational changes in the 195 kDa 'nicked CAD' species, which can be retarded by the nucleotides ATP or UTP.

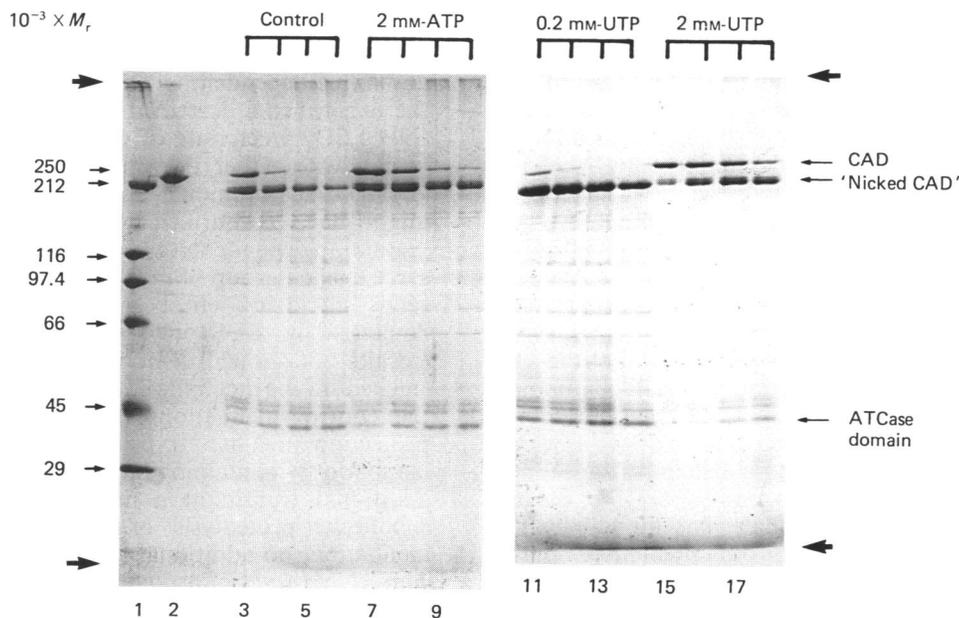
#### Proteolysis of CAD in the presence of nucleotides

Despite their different specificities, both elastase and trypsin cleave CAD at exposed inter-domain sites when



**Fig. 4. Products of limited proteolysis of CAD by elastase at 37 °C**

CAD (40  $\mu\text{g}$ ) was incubated for 10 min at 37 °C in 0.1 M-Hepes, pH 7.4, containing 4 mM- $\text{MgCl}_2$  and nucleotides as detailed in the text before the addition of 0.3  $\mu\text{g}$  of elastase. Samples were withdrawn and processed for electrophoresis after 15, 30, 45 and 60 min digestion. Lane 1, undigested control incubation of CAD. Lane 2, protein markers: 212 kDa (myosin heavy chain), 116 kDa (*Escherichia coli*  $\beta$ -galactosidase), 97.4 kDa (rabbit muscle phosphorylase *b*), 66 kDa (bovine serum albumin), 45 kDa (hen ovalbumin) and 29 kDa (bovine carbonic anhydrase).  $M_r$  markers to the right of the gel correspond to a similar mixture, with rabbit mammary fatty acid synthase (250 kDa) replacing myosin. Groups of four lanes from left to right correspond to timed samples removed from digestion conditions as follows: 3–6, control incubation; 7–10, 2 mM-ATP; 11–14, 0.2 mM-UTP; 15–18, 2 mM-UTP; all incubations contained 4 mM- $\text{MgCl}_2$ . Thick arrows indicate top of gel and dye front.



**Fig. 5. Products of limited proteolysis of CAD by trypsin at 37 °C**

Incubation and digestion conditions were identical with those described in the legend to Fig. 4, except that 57 ng of trypsin was added to initiate the digestion. Lane 1,  $M_r$  markers as in Fig. 4. Lane 2, undigested control incubation of CAD. Groups of four lanes correspond to samples withdrawn at 15, 30, 45 and 60 min from incubation conditions as follows: 3–6, control; 7–10, 2 mM-ATP; 11–14, 0.2 mM-UTP; 15–18, 2 mM-UTP; all incubations also contained 4 mM- $\text{MgCl}_2$ . The positions of CAD, 'nicked CAD' and the ATCase domain are indicated.

used at low concentrations. In the present work, trypsin was used at much lower concentrations than previously [6], and was found to generate a range of polypeptide products similar in many respects to the products of limited digestion by elastase [5], but with slight differences, for example in the size of the domain liberated by the first cleavage step. CAD was preincubated for 10 min at 37 °C before addition of proteinases, in order to assess whether the differences in enzyme lability between the control incubations and those containing nucleotides were accompanied by conformational changes in CAD.

In common with previous work [5, 6], an apparent molecular mass of 210–220 kDa was obtained for CAD in the undigested control lane of the SDS/polyacrylamide gel when the mixture of marker proteins containing myosin (212 kDa) was used for comparison (see Figs. 4 and 5). In this work, a marker mixture containing rabbit fatty acid synthase (250 kDa) was also used, and from analysis of four separate electrophoresis gels a subunit mass of 235–240 kDa for CAD was obtained, which is consistent with the relative sizes of the digestion products obtained in the present and previous work [5].

Elastase digestion rapidly generated a range of polypeptides in the control incubation (Fig. 4). The sizes of the peptides and the order of their release were compatible with the scheme [5] in which the earliest step in cleavage of CAD by elastase liberated the C-terminal ATCase domain from the rest of the molecule (195 kDa), although the smaller fragment ran on SDS/polyacrylamide-gel electrophoresis with an apparent size of 46 kDa in the present work.

Preincubation with 2 mM-ATP, 5 mM-p[CH<sub>2</sub>]ppA (results not shown) and 2 mM-UTP retarded the action of elastase on CAD, whereas in 10 mM-ATP the CAD polypeptide was totally resistant to elastase (results not shown). After incubation in 0.2 mM-UTP, the stained band corresponding to CAD disappeared at approximately the same rate as in the control lanes, but the 195 kDa polypeptide accumulated to a greater extent, indicating that at the lower concentration of UTP only the 'nicked CAD' was protected from further proteolysis to liberate the DHOase domain.

It was found that approx. 1:750 (w/w) trypsin:CAD digested CAD more thoroughly than elastase at 1:150 (w/w) ratio. Under these conditions, as shown in the control lanes of Fig. 5, the intermediate-sized fragments differed principally by the presence of a peptide corresponding to 76 kDa in the tryptic digest only. In SDS/polyacrylamide-gel-electrophoretic analysis of several tryptic mixtures, the 'nicked CAD' of 195 kDa formed a closely spaced triplet of bands, and the ATCase domain also was represented by a group of three bands corresponding to molecular sizes of 43, 42 and 39 kDa. It would seem that, unlike the apparently unique initial cleavage of the linking region by elastase, three or more equally trypsin-sensitive sequences are accessible between the ATCase domain and the rest of the CAD molecule, leading to the production of at least three pairs of polypeptides. Subsequent cleavage of each of the 'nicked CAD' polypeptides may occur at a unique trypsin-sensitive site, since only a triplet of bands corresponded to the 155 kDa polypeptide generated by the removal of the DHOase domain.

In the presence of ATP or p[CH<sub>2</sub>]ppA, the tryptic digestion products accumulated more slowly than in the

control lanes (Fig. 5). The bands corresponding to molecular masses of 76 kDa and 155 kDa were completely absent from samples of the incubation with 10 mM-ATP (results not shown). The first cleavage step was retarded by 2 mM-ATP and 5 mM-p[CH<sub>2</sub>]ppA, and, among the intermediate-sized products, less of the 76 kDa peptide was produced, and a new peptide at 66 kDa was observed.

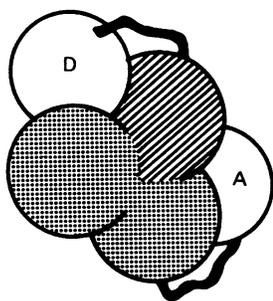
The effects of UTP on tryptic digestion of CAD were qualitatively similar but more marked than in the digestion by elastase. The tryptic cleavage which released the ATCase domain was retarded to a similar degree by 2 mM-ATP or 2 mM-UTP, but in the latter incubation the further processing of the 195 kDa product was completely inhibited. At the lower UTP concentration (0.2 mM) the first cleavage took place at the same rate as in the control incubations, but there was a very marked accumulation of the 195 kDa 'nicked CAD' even after 1 h, when all the CAD had been digested. The protective effect of 0.2 mM-UTP in tryptic digests has been successfully applied (E. A. Carrey, unpublished work) to the preparation of active forms of both 'nicked CAD' and a 43 kDa ATCase domain, which were separated by slight modifications of the method of Grayson & Evans [7].

In similar studies (results not shown), 2 mM-UTP and 2–10 mM-ATP were not additive in promoting the accumulation of the undigested CAD, suggesting that at these concentrations both nucleotides stabilize the intact molecule through similar mechanisms. The 'nicked CAD' accumulated to the same extents found in incubations with UTP alone, suggesting in turn that ATP does not interfere with the protection of the 195 kDa fragment by UTP.

## DISCUSSION

The new assay protocols allowed the production of carbamoyl phosphate to be measured in the absence of sodium aspartate (assay system 1). In addition, the NH<sub>3</sub>- and glutamine-dependent activities of CPSaseII during incubation at 37 °C were studied separately. The differences in the thermal-inactivation profiles suggested that the glutaminase activity is found in a discretely folded domain independent of the NH<sub>3</sub>-dependent synthetase, and that sodium aspartate stabilized the more labile glutaminase domain. Both activities decreased less rapidly in 2–10 mM-MgATP, but only the NH<sub>3</sub>-dependent synthetase was stabilized by the negative effector UTP. Neither enzyme activity was protected by the non-hydrolysable analogue p[CH<sub>2</sub>]ppA or by the positive allosteric effector PPRibP.

In view of the close relationships between the CPSases, demonstrated by sequence comparisons [10, 11] and immunochemically [9], it is possible that the central CPSaseII moiety within CAD resembles CPSaseI in having a glutaminase domain N-terminal to a pair of nucleotide-binding domains which catalyse the synthase reaction [11]. The linear sequence of domains in CAD would thus be DHOase–glutaminase–(duplicate synthase domains)–ATCase, but the spatial arrangement of the domains is not known. A model is suggested in Fig. 6, in which flexible linking regions, already known from limited proteolysis [5, 6], allow the DHOase and ATCase domains to pack close to the CPSase moiety, with the ATCase domain adjacent to the glutaminase domain. Since the lability of the glutaminase domain would limit



**Fig. 6. Model for the conformation of CAD**

In this model it is proposed that ATCase (A) and DHOase (D) activities reside in single globular domains at the C-terminus and N-terminus respectively of the CAD polypeptide, and the central CPSase moiety of 155 kDa comprises three domains of approximately equal size. The flexible linking regions (thick black lines) allow the glutaminase domain (hatched) and the two nucleotide-binding domains (stippled) to contact the A and D domains. Evidence is given in the text that ligand-induced changes in the ATCase or nucleotide-binding domains stabilize the conformation of the glutaminase domain.

the supply of ammonia groups to the synthetase, the binding of substrates (MgATP and aspartate) to adjacent domains may be important in regulating the activity of the glutaminase through its thermal stability. In addition, the juxtaposition of the glutaminase, CPSase and ATCase active sites in such an arrangement could be very favourable for the channelling of the ammonia group from glutamine into carbamoylaspartate.

The duplicated (synthase) regions in the C-terminal part of the rat mitochondrial CPSaseI gene [11] each contain two nucleotide-binding sequences. An ATP molecule may bind to the more closely conserved site on each domain, possibly with co-operative interactions between domains, and MgUTP may bind at a separate site. Alternatively, it has been suggested, on the basis of kinetic evidence from CPSaseII [24], that the co-operativity for MgATP arises from interactions between separate molecules of CAD, and that MgUTP is a competitive inhibitor for ATP. When the conformation of CAD was probed through the proteolysis of the inter-domain regions, it was clear that MgATP and MgUTP stabilized the molecule against proteolysis through distinct mechanisms. In the presence of 0.2 mM-MgUTP, only the CPSase-DHOase linking region was protected against proteolysis (see Fig. 6) possibly arising from conformational changes in the nucleotide-binding domain adjacent to the DHOase domain. In 2 mM-MgUTP there appeared to be an additional conformational effect which restricted proteolysis of the CPSase-ATCase linking region, similar to the effect of 2 mM-MgATP, and, since these effects were not additive, it is probable that at higher concentrations MgUTP is able to compete for one of the binding sites available to MgATP. Unlike MgATP, however, 2 mM-MgUTP did not prevent the inactivation of the glutaminase activity, indicating that a different conformational change in the CPSase domain was transmitted to the glutaminase domain. By binding to the central CPSase moiety, each nucleotide retarded the unfolding as measured by proteolysis and the inactivation of the  $\text{NH}_3$ -dependent synthetase at 37 °C.

The concentrations of ATP and UTP in mammalian cells have been estimated at 2–3 mM and 0.3 mM respectively [25]. The results described here suggest that under physiological conditions both nucleotides could be important in maintaining the stability and relative topology of the domains in CAD. Both the CPSase and the glutaminase activities would be less labile at 37 °C through the binding of ATP, which would also restrict the accessibility of the CPSase-ATCase linking region to proteolytic attack. Little additional thermal stabilization would be provided by UTP, which in any case is an inhibitor of CPSaseII, but physiological concentrations were effective in preventing further proteolysis of any 'nicked CAD' which could be present. The latter effect could be important in preventing degradation of the 193 kDa mutant enzyme found in some lines of Chinese hamster ovary cells when the cells are provided with exogenous uridine [26].

This work was supported by a project grant from the Medical Research Council. I am grateful to Professor P. Cohen and Dr. D. G. Hardie for helpful discussions and encouragement, and to Professor G. R. Stark and Dr. D. R. Evans for help in establishing the overproducing cell line.

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Received 10 October 1985/30 December 1985; accepted 17 January 1986