Physical Location of the Site for *N*-Acetyl-L-glutamate, the Allosteric Activator of Carbamoyl Phosphate Synthetase, in the 20-Kilodalton COOH-Terminal Domain†

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ABSTRACT: Mammalian liver mitochondrial carbamoyl phosphate synthetase, a polypeptide of 160 kDa, is activated allosterically by *N*-acetyl-L-glutamate. The analogue of this activator *N*-(chloroacetyl)-L-[^{14}C]glutamate has been found to serve as a photoaffinity label for this enzyme. The specificity was demonstrated by the drastic reduction in the radioactivity bound to the protein when (a) an excess of unlabeled acetylglutamate was present during the irradiation and (b) the enzyme was replaced by pyruvate kinase, an enzyme that is not affected by acetylglutamate. The labeling was due to the photoactivation of the chloroacetyl group since there was no labeling under equal conditions with acetyl[^{14}C]glutamate. To localize the binding site, limited proteolysis was used. Trypsin cleaves carbamoyl phosphate synthetase into complementary NH_2- and COOH-terminal fragments of about 140 and 20 kDa, respectively [Powers-Lee, S. G., & Corina, K. (1986) *J. Biol. Chem. 261*, 15349-15352], but only the latter was found to be labeled. Similarly, of the various fragments generated by elastase, only two, of 20 and 120 kDa, contain the COOH terminus [see Powers-Lee and Corina (1986) above] and were found to be labeled. Thus, the binding site for acetylglutamate is within 20 kDa from the COOH terminus. This excludes the possibility that the acetylglutamate binding site evolved from an ancestral substrate site for glutamine: this substrate binds to the small subunit of the *Escherichia coli* enzyme, which is homologous to the NH_2-terminal domain of the rat liver enzyme. Exhaustive tryptic digestion of photolabeled carbamoyl phosphate synthetase yielded a single radioactive peak, suggesting that the labeling is restricted to a single minimal tryptic peptide.

Carbamoyl phosphate synthetase I (ammonia) (CPS I;[1] EC 6.3.4.16), a polypeptide of 160 kDa (Nyunoya et al., 1985), catalyzes the first step of the urea cycle:

$$2ATP + NH_3 + HCO_3^- \rightarrow 2ADP + P_i + \text{carbamoyl phosphate}$$

The enzyme is activated >50-fold by *N*-acetyl-L-glutamate (Rubio et al., 1983a). This confers on acetylglutamate an obvious potentiality to control the flux through the urea cycle.

Activation by acetylglutamate involves reversible, noncovalent binding of a molecule of the activator per molecule of the polypeptide (Alonso & Rubio, 1983). Several cryoprotectant agents chemically unrelated to acetylglutamate, at high concentrations, were shown to mimic acetylglutamate in activating the enzyme (Rubio et al., 1983b). This strongly supports the long-held view that acetylglutamate activates by inducing an active conformation of the enzyme [reviewed in Rubio et al. (1983b)]. In this active form essential SH groups (Marshall et al., 1961; Novoa et al., 1966; Marshall & Fahien, 1985) and the binding site for the ATP molecule that yields P_i (Rubio et al., 1983a) are exposed, and the enzyme becomes highly susceptible to thermal (Caravaca & Grisolia, 1959; Fahien et al., 1964), oxidative (Alonso & Rubio, 1987), and proteolytic (Guadalajara et al., 1987; Marshall & Fahien, 1988) inactivation. Limited proteolysis reveals that the enzyme is composed, from the N-terminus, of four domains of about 40, 40, 60, and 20 kDa (Powers-Lee & Corina, 1986, 1987; Evans & Balon, 1988; Marshall & Fahien, 1988). Acetylglutamate increases approximately 9-fold the rate of cleavage of the hinge connecting the COOH-terminal domain of 20 kDa to the remainder of the enzyme molecule (Marshall & Fahien, 1988), and it also appeared to protect in some experiments this COOH-terminal domain after cleavage (Powers-Lee & Corina, 1987).

To understand the mechanism of activation of the synthetase, it is essential to determine the location of the binding site for acetylglutamate in the enzyme. This is also relevant because other carbamoyl phosphate synthetases are susceptible to a number of positive modulators although not to acetylglutamate (Meister & Powers, 1978; Mori & Tatibana, 1978); in view of the homologies between the various synthetases for which the sequences are known [see Nyunoya et al. (1985)], it is probable that modulatory sites in different synthetases are derived from a single ancestral site.

In this paper we demonstrate, using a combination of limited proteolysis and photoaffinity labeling with *N*-(chloroacetyl)-L-glutamate, an active (Grisolía & Cohen, 1953) and photosensitive (Bosch et al., 1985) analogue of acetylglutamate, that this activator binds to the 20-kDa COOH-terminal domain of rat liver carbamyl phosphate synthetase.

Materials and Methods

Chemicals and Enzymes. Mitochondrial carbamoyl phosphate synthetase was purified from rat liver (Guadalajara et al., 1987). Ammonium sulfate was removed from the enzyme preparation by centrifugal gel filtration (Penefsky, 1977)

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[1] Abbreviations: AG or acetylglutamate, *N*-acetyl-L-glutamate; (chloroacetyl)glutamate, *N*-(chloroacetyl)-L-glutamate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CPS I, carbamoyl phosphate synthetase (ammonia); ATP_A, the ATP molecule that yields P_i in the enzyme reaction; ATP_B, the molecule that provides the phosphoryl group of carbamoyl phosphate.

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through Sephadex G-25. Pyruvate kinase (rabbit muscle, salt free) and elastase (type IV, porcine pancreas) were from Sigma. Trypsin (bovine pancreas) was from Boehringer or, for tryptic maps, from Sigma (TPCK treated).

N-(Chloroacetyl)-L-[^{14}C]glutamate (285 Ci/mol) was synthesized from chloroacetyl chloride (Aldrich) and L-[U-^{14}C]glutamate (from Amersham) by scaling down the procedure of Fischer et al. (1909). Unreacted glutamate was removed by passage of the mixture through a column (0.8 × 4 cm) of AG 50W×8 (H^+; 200–400 mesh). The effluent and a 5-mL wash with 0.1 M HCl were combined and evaporated, and the residue was dissolved in water and applied to a column (1.5 × 5.6 cm) of AG 1×8 (formate; 200–400 mesh). After washing in succession with 200 mL of 0.1 M formate and 180 mL of 0.3 M formate brought to pH 3.0 with pyridine, (chloroacetyl)[^{14}C]glutamate was eluted with 80 mL of 4 M formic acid. The solvent was flash-evaporated, and (chloroacetyl)glutamate was stored at −20 °C as a 0.15 mM solution in water. Purity, tested by thin-layer chromatography (cellulose plates, DC-Alufolien, Merck; solvent, butanol/acetic acid/water, 6:1:2; R_f = 0.81) was >95%.

N-Acetyl-L-[^{14}C]glutamate (285 Ci/mol) was synthesized from L-[U-^{14}C]glutamate as previously described (Alonso & Rubio, 1983).

Photoaffinity Labeling of Carbamoyl Phosphate Synthetase. The enzyme (2–3 mg/mL) in a solution of 50 mM Tris-HCl, pH 7.2, 50 mM KCl, 1 mM dithioerythritol, and 20% (v/v) glycerol was incubated for 15 min at 37 °C with 15 μM (chloroacetyl)[^{14}C]glutamate. The solution (50–200 μL) was then transferred to a square quartz cuvette of the type used for fluorescent measurements (all sides transparent) with an inner rectangular sample chamber of 1 × 0.2 cm. This cuvette was placed in a 10-mL Pyrex beaker which was attached to the outer wall of a cylindrical, water-thermostated, immersion well quartz reactor (Bosch et al., 1975) fitted with a 125-W medium-pressure mercury lamp. The cuvette was positioned with the long side of the inner chamber perpendicular to the light, so that the optical pathway at the center of the cuvette was 0.2 cm. The mixture was irradiated for 10 min at 25 °C, and the magnitude of labeling was determined by precipitation with 10% trichloroacetic acid.

Limited Proteolysis. The photolabeled enzyme (1.2 mg/mL) was incubated at 37 °C with trypsin (4 μg/mL) or elastase (25 μg/mL) in a solution containing 30 mM Tris-HCl, pH 7.5, 8% (v/v) glycerol, 1.4 mM dithioerythritol, 36 mM KCl, 20 mM NaCl, 10 mM acetylglutamate, and, when elastase was used, 10 mM ATP and 20 mM $MgSO_4$. At the indicated periods of incubation 40-μL samples were added to 60 μL of a solution at 100 °C containing 0.23 M Tris-HCl, pH 6.8, 4% (w/v) SDS, and 18% (v/v) 2-mercaptoethanol; boiling was continued for 5 min. After cooling, the samples were desalted by centrifugal gel filtration (Penefsky, 1977) through Sephadex G-25 fine, equilibrated with 0.125 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, and 8% (v/v) glycerol. They were then subjected to SDS–PAGE (Laemmli, 1970) in 5–15% exponential gradient gel slabs. The gels were stained with Coomasie brilliant blue R-250 (Sigma) or processed and dried for fluorography (Bonner & Laskey, 1974), which was carried out at −70 °C with preflashed film (Hyperfilm-MP, Amersham). Alternatively, the Coomassie-stained bands were excised from the gel and dissolved in 20% H_2O_2 and 40% $HClO_4$, and the radioactivity was determined.

Tryptic Peptide Mapping. The photolabeled enzyme solution was diluted with 2.5 volumes of 6 M guanidine hydrochloride in 0.5 M Tris-HCl, pH 8.5, and was subjected to centrifugal gel filtration through Sephadex G-25 equilibrated with the guanidine–Tris solution. Reduction, carboxymethylation, tryptic digestion, and HPLC analysis were carried out as described by Mayes (1984). Urea (2 M) had to be included at the digestion step to solubilize the protein. For HPLC, a Vydac reverse-phase 5-μm C-18 column (type 218TP54, 0.46 × 25 cm) and a 1-h linear gradient from 0 to 57% acetonitrile (0.1% trifluoroacetic acid present) were used, at a flow rate of 1 mL/min. The optical absorption of the effluent at 214 and 280 nm was monitored, and 1-mL fractions were collected for assay of radioactivity.

Table I: Photolabeling of Carbamoyl Phosphate Synthetase by (Chloroacetyl)[^{14}C]glutamate[a]

components of the mixture	radioactivity precipitated with trichloroacetic acid (dpm)
carbamoyl phosphate synthetase + (chloroacetyl)[^{14}C]glutamate (15 μM)	5390
carbamoyl phosphate synthetase + (chloroacetyl)[^{14}C]glutamate (15 μM) + acetylglutamate (1 mM)	731
pyruvate kinase + (chloroacetyl)[^{14}C]glutamate (15 μM)	457
carbamoyl phosphate synthetase + acetyl[^{14}C]glutamate (15 μM)	25

[a] Irradiation of the synthetase or, when specified, of pyruvate kinase (both at 3 mg/mL) in the presence of the indicated additions was carried out as described under Materials and Methods. The radioactivity given is that precipitated from a 5-μL sample from each incubation. Total radioactivity (soluble + precipitated) in these 5-μL samples was 46 600 ± 1860 dpm.

Results

Photolabeling of the Enzyme. Table I shows that, upon irradiation of a mixture of (chloroacetyl)[^{14}C]glutamate and carbamoyl phosphate synthetase, a significant proportion of the radioactivity was precipitated with trichloroacetic acid, indicating labeling of the enzyme. This was demonstrated also by gel filtration through Sephadex G-25 (results not shown) and by polyacrylamide gel electrophoresis in the presence of SDS (see below). Addition of unlabeled acetylglutamate or replacement of carbamoyl phosphate synthetase by pyruvate kinase, an enzyme that is not affected by acetylglutamate, drastically decreased the radioactivity precipitated by trichloroacetic acid, indicating that the labeling of the synthetase was specific (Table I). That the Cl group in (chloroacetyl)-glutamate was responsible for the photolabeling of the enzyme was shown by replacing (chloroacetyl)[^{14}C]glutamate by acetyl[^{14}C]glutamate: essentially no radioactivity was precipitated with trichloroacetic acid in this case (Table I).

Limited Proteolysis. Trypsin splits first the 160-kDa polypeptide that constitutes carbamoyl phosphate synthetase at a point about 20 kDa from the COOH terminus (Powers-Lee & Corina, 1986, 1987; Evans & Balon, 1988; Marshall & Fahien, 1988), and then the resulting fragment of 140 kDa is split at a second point, yielding fragments of approximately 80 and 60 kDa (Figure 1). Figure 2 (left) illustrates the results of the electrophoretic analysis of the photolabeled enzyme after treatment for various periods with trypsin. Irradiation damages some enzyme molecules, and this is reflected in the multiplicity of polypeptides of <160 kDa seen at time zero of treatment with trypsin (compare with the nonirradiated enzyme, Figure 2). Nevertheless, most of the protein migrates, as expected, as a polypeptide of 160 kDa. With time of treatment with trypsin the fragments of 140, 80, and 60 kDa accumulate and predominate at the longer periods of proteo-

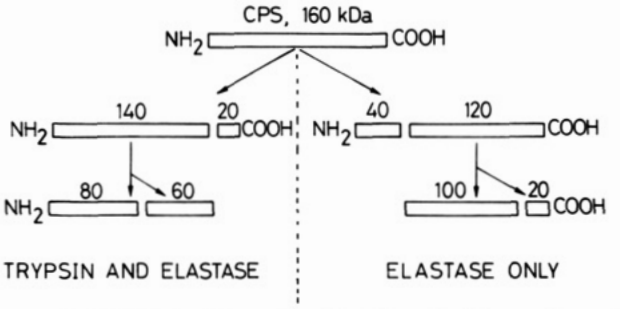

FIGURE 1: Initial steps of the routes of digestion of rat liver carbamoyl phosphate synthetase by elastase and trypsin. Derived from data in Powers-Lee and Corina (1986, 1987), Evans and Balon (1988), and Marshall and Fahien (1988), and from unpublished results from this laboratory. The approximate size of each fragment is given in kilodaltons.

lytic treatment. The COOH-terminal fragment of 20 kDa accumulates to a limited extent only, confirming an earlier report that this fragment is proteolyzed to smaller peptides that are not retained in the gel (Powers-Lee & Corina, 1986).

Fluorographic analysis (Figure 2, right) revealed that the enzyme (the band of 160 kDa) was labeled and that the radioactivity and the amount of protein in the band of 160 kDa (Figure 3, top) decreased in parallel with time of treatment. None of the bands of 140, 80, or 60 kDa were labeled. This excludes that (chloroacetyl)[^{14}C]glutamate was cross-linked to the NH_2-terminal moiety of 140 kDa (Figure 1) and indicates that the binding site for acetylglutamate is at the 20-kDa COOH-terminal domain. In agreement with this idea the fluorography reveals (Figure 2) the appearance of a labeled fragment of about 20 kDa, which is degraded and lost at the longer periods of incubation with trypsin.

An independent confirmation that (chloroacetyl)[^{14}C]-glutamate was cross-linked exclusively to the COOH-terminal domain of 20 kDa was obtained with elastase (Figure 4). This enzyme, in addition to cleaving carbamoyl phosphate synthetase at the same places as trypsin, has an extra cleavage point at 40 kDa from the NH_2 terminus (Powers-Lee & Corina, 1986, 1987; Evans & Balon, 1988; Marshall & Fahien, 1988) and thus generates a 120-kDa fragment that includes the 20-kDa COOH-terminal domain (Figure 1). If, as indicated by the results with trypsin, the binding site for acetylglutamate is at this domain, the fragment of 120 kDa should be labeled (Figure 1). This is confirmed in Figure 4, which demonstrates that, although the fragment of 120 kDa only accumulated in moderate amount, compared with other fragments (Figure 4, left) it was the only fragment generated by elastase that was labeled in addition to that of 20 kDa (Figure 4, right). This was corroborated by determination of radioactivity of the excised Coomassie-stained bands (results not shown). Figure 3 (bottom) demonstrates that, as with trypsin, digestion by elastase decreased essentially in the same proportion protein and radioactivity in the band of 160 kDa.

Tryptic Map. Exhaustive tryptic digestion of carbamoyl phosphate synthetase is expected to generate 19 fragments from the 20-kDa COOH-terminal domain. However, in tryptic maps of the labeled enzyme only a single radioactive peak was observed (Figure 5). This further supports the specificity of the labeling and suggests that (chloroacetyl)[^{14}C]glutamate is cross-linked to a single minimal tryptic peptide.

Discussion

From a combination of sequence data (Nyunoya et al. 1985), previous results of limited proteolysis (Powers-Lee & Corina, 1986, 1987; Evans & Balon, 1988; Marshall & Fahien, 1988), and our own findings (unpublished data), it appears that mammalian carbamoyl phosphate synthetase is composed of four main globular domains of about 40, 40, 60, and 20 kDa, counting from the NH_2 terminus. The NH_2-terminal domain of 40 kDa is homologous to the glutaminase subunit of carbamoyl phosphate synthetase from *Escherichia coli* (Nyunoya et al., 1985). However, the mammalian enzyme is unable to use glutamine (Kerson & Appel, 1968) and the role of this domain in the enzyme is uncertain. Binding of inert glutamine analogues to the glutamine site in the small subunit of carbamoyl phosphate synthetase from *E. coli* increases the ability of the large subunit to cleave ATP and to bind or use ammonia (Meister & Powers, 1978). Therefore, an attractive possibility was that the substrate site for glutamine had evolved to accommodate acetylglutamate in the mammalian enzyme. Our results, however, rule out this hypothesis, for they demonstrate that acetylglutamate binds to the 20-kDa COOH-terminal domain which is at the opposite end of the polypeptide chain. This domain is within the moiety of mammalian carbamoyl phosphate synthetase that is homologous to the large subunit of the *E. coli* enzyme (Nyunoya et al., 1985). In this microorganism, the large subunit is responsible for carbamoyl phosphate synthesis from NH_3, and it also contains the sites for the positive effectors ornithine and IMP (Meister & Powers, 1978). Ornithine in the *E. coli* enzyme (Meister &

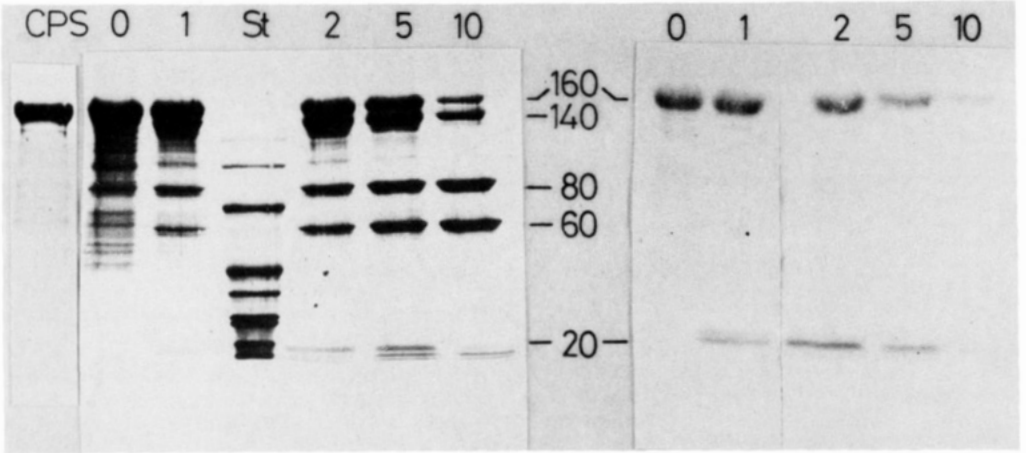

FIGURE 2: Time course of trypsin digestion of photolabeled carbamoyl phosphate synthetase. Left, Coomassie staining of SDS–PAGE. Right, fluorography. CPS refers to the nonirradiated enzyme. St refers to molecular weight markers (M_r of 116 000, 97 400, 66 000, 45 000, 36 000, 29 000, 24 000, 20 000, and 14 200). The minutes of incubation with trypsin are given at the top, and the molecular weights of the fragments are given on the ordinate.

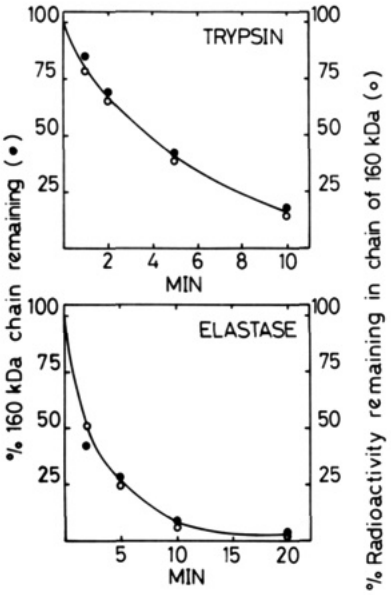

FIGURE 3: Protein and radioactivity in the band of 160 kDa with time of incubation with trypsin and elastase. The amount of protein was determined by scanning the Coomassie-stained wet gels by using an LKB laser-scanning densitometer. The amount of radioactivity was determined by scanning densitometry of the developed fluorography film. Results are expressed as a percentage of the amounts of protein (●) and radioactivity (○) at time zero of proteolytic treatment.

Powers, 1978) and acetylglutamate in the mammalian enzyme (Rubio et al., 1983a) increase the apparent affinity for ATP, and glycerol partly replaces these activators in both enzymes (Rubio & Llorente, 1982; Rubio et al., 1983b). With carbamoyl phosphate synthetase II (the enzyme that catalyzes the first step of pyrimidine synthesis in mammals) phosphoribosyl pyrophosphate, another positive effector, also increases the apparent affinity for ATP and again is partly replaced by glycerol (Ishida et al., 1977). Thus, activation of the synthetases by these effectors exhibits similarities that may reflect a common mechanism. Therefore, there may be a topographically equivalent site that would have evolved to yield the particular specificity for each enzyme.

Binding of acetylglutamate to the 20-kDa COOH-terminal domain greatly increases the susceptibility of rat liver carbamoyl phosphate synthetase to proteolytic attack of the region that joins the 20-kDa COOH-terminal domain to the rest of the molecule (Marshall & Fahien, 1988); thus, a region about 20 kDa from the COOH terminus is exposed. ATP protects this region from proteolysis (Powers-Lee & Corina, 1986; Marshall & Fahien, 1988). Binding of acetylglutamate is also known to allow access of ATP to the site for the molecule of ATP_A (ATP_A yields P_i in the reaction) (Rubio et al., 1983a). Thus, this point of proteolytic cleavage is likely to be at or close to the binding site for ATP_A. Indeed, one of two highly conserved putative ATP binding sequences detected by Nyunoya et al. (1985) is located at 22 kDa from the COOH terminus. The site for ATP_A is involved in making the mixed carbonic–phosphoric anhydride, which is highly susceptible to nucleophilic attack and must be excluded from water (Rubio & Grisolia, 1977; Rubio et al., 1981). The COOH-terminal domain of 20 kDa may provide protection by shielding the site for ATP_A. We propose that acetylglutamate binding relaxes the hinge region between the 20-kDa COOH-terminal domain and the rest of the molecule, allowing movement to occur, thus exposing the site for ATP_A. Conformational changes were detected kinetically as a part of the normal enzyme reaction (Britton et al., 1979) and may reflect the changes at this COOH-terminal domain. The existence of changes in conformation of the site for acetylglutamate with catalysis and with binding of ATP_A is strongly supported also by the drastic increase in the affinity for acetylglutamate that is induced by addition of ATP at concentrations adequate to saturate the ATP_A site, and by the decrease in affinity for acetylglutamate that is induced by catalytic cycling of the enzyme in the presence of ammonia (Alonso & Rubio, 1983).

The present results provide a function for the 20-kDa COOH-terminal domain of the mammalian enzyme. As already discussed, the 60-kDa adjacent domain appears to contain the ATP_A binding site. Sequence analysis provides evidence that the 40-kDa domain that precedes that of 60 kDa is also involved in binding of ATP (Nyunoya et al., 1985; Powers-Lee & Corina, 1987), and this may represent the ATP_B binding site (ATP_B provides the phosphoryl group of carbamoyl phosphate). Further, both domains were photolabeled with 8-azido-ATP (Powers-Lee & Corina, 1987). The function of the remaining 40-kDa NH_2-terminal domain remains to be established. Since the homologous small subunit in the *E. coli* enzyme influences the efficiency with which the large subunit uses ammonia (Meister & Powers, 1976; Rubino et al., 1987), this NH_2-terminal domain may be involved in the determination of the high affinity for NH_3 of the mammalian enzyme. To summarize, our data provide strong ev-

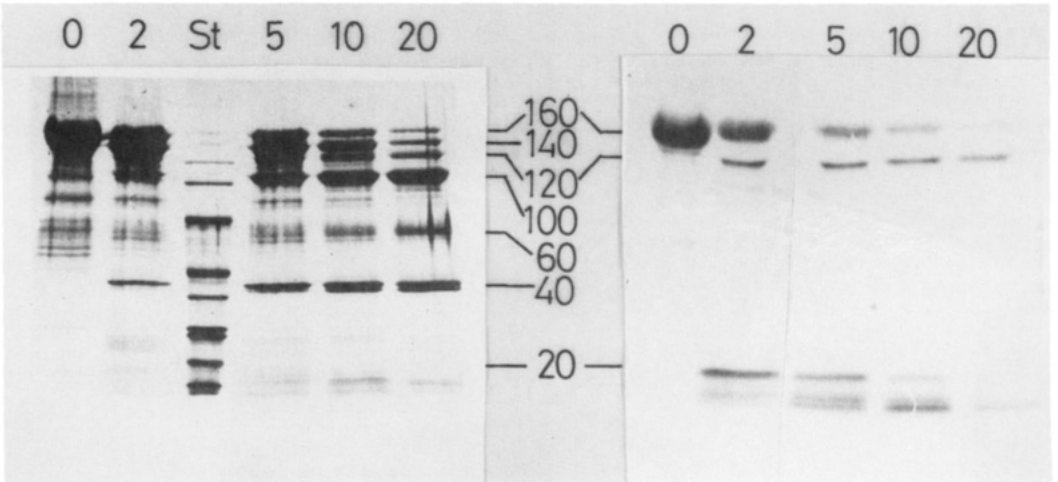

FIGURE 4: Time course of elastase digestion of photolabeled carbamoyl phosphate synthetase. Left, Coomassie staining of SDS–PAGE. Right, fluorography. St refers to molecular weight markers (M_r of 116 000, 97 400, 66 000, 45 000, 36 000, 29 000, 24 000, 20 000, and 14 200). The minutes of incubation with elastase are given at the top, and the molecular weights of the fragments are given on the ordinate.

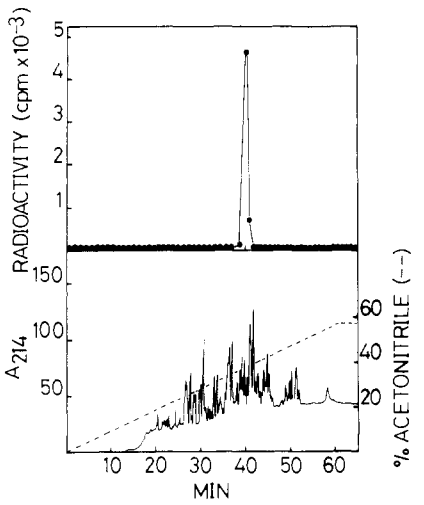

FIGURE 5: Tryptic map of photolabeled carbamoyl phosphate synthetase. For details see Materials and Methods.

idence that the COOH-terminal domain of 20 kDa is responsible for binding of acetylglutamate. We propose as a working hypothesis that an equivalent domain in other carbamoyl phosphate synthetases is involved in binding of positive effectors.

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Registry No. CPS I, 9026-23-7; AG, 1188-37-0; (chloroacetyl)-glutamate, 56576-86-4.

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