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Domain Structure of the Large Subunit of *Escherichia coli* Carbamoyl Phosphate Synthetase. Location of the Binding Site for the Allosteric Inhibitor UMP in the COOH-Terminal Domain†

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ABSTRACT: The large subunit of *Escherichia coli* carbamoyl phosphate synthetase (a polypeptide of 117.7 kDa that consists of two homologous halves) is responsible for carbamoyl phosphate synthesis from NH_3 and for the binding of the allosteric activators ornithine and IMP and of the inhibitor UMP. Elastase, trypsin, and chymotrypsin inactivate the enzyme and cleave the large subunit at a site approximately 15 kDa from the COOH terminus (demonstrated by NH_2-terminal sequencing). UMP, IMP, and ornithine prevent this cleavage and the inactivation. Upon irradiation with ultraviolet light in the presence of [^{14}C]UMP, the large subunit is labeled selectively and specifically. The labeling is inhibited by ornithine and IMP. Cleavage of the 15-kDa COOH-terminal region by prior treatment of the enzyme with trypsin prevents the labeling on subsequent irradiation with [^{14}C]UMP. The [^{14}C]UMP-labeled large subunit is resistant to proteolytic cleavage, but if it is treated with SDS the resistance is lost, indicating that UMP is cross-linked to its binding site and that the protection is due to conformational factors. In the presence of SDS, the labeled large subunit is cleaved by trypsin or by V8 staphylococcal protease at a site located 15 or 25 kDa, respectively, from the COOH terminus (shown by NH_2-terminal sequencing), and only the 15- or 25-kDa fragments are labeled. Similarly, upon cleavage of the aspartyl–prolyl bonds of the [^{14}C]UMP-labeled enzyme with 70% formic acid, labeling was found only in the 18.5-kDa fragment that contains the COOH terminus of the subunit. Thus, UMP binds to the COOH-terminal domain. Since the binding sites for IMP and UMP overlap, most probably IMP also binds in this domain. The protection from proteolysis by ornithine suggests that ornithine binds in the same domain. Acetylglutamate (the allosteric activator of the ureotelic enzyme) binds to the homologous COOH-terminal domain of the rat liver enzyme [Rodriguez-Aparicio, L., et al. (1989) *Biochemistry 28*, 3070–3074]. Thus, the COOH-terminal domain appears to be the regulatory domain of the carbamoyl phosphate synthetases. To account for the effects of the allosteric effectors on the binding of ATP, we propose a scheme where the two halves of the large subunit form a pseudohomodimer by complementary isologous association, thus placing the NH_2 half, which is involved in the binding of the molecule of ATP that yields P_i, close to the regulatory domain.

Carbamoyl phosphate synthetase catalyzes the first committed step in the urea, arginine, and pyrimidine biosynthesis pathways. Carbamoyl phosphate synthetase is therefore a good candidate for metabolic control, and indeed, the enzyme from a number of sources is subject to allosteric regulation [reviewed in Rubio et al. (1983b)]. The nature of the effectors depends on the physiological function of the synthetase. For example, the ureotelic enzyme is activated by *N*-acetyl-L-glutamate (acetylglutamate)[1] (Hall et al., 1958), whereas the *Escherichia coli* enzyme, which is involved in both pyrimidine and arginine synthesis, is activated by IMP and ornithine and inhibited by UMP (Meister & Powers, 1978).

The activation of the rat liver mitochondrial enzyme (carbamoyl phosphate synthetase I) has been investigated extensively (Alonso & Rubio, 1983; Rubio et al., 1983a; Guadalajara et al., 1987; Britton & Rubio, 1988; Rodriguez-Aparicio et al., 1989; Britton et al., 1990). We have shown that a single molecule of acetylglutamate is bound per enzyme molecule (M_r 160000). The affinity for acetylglutamate is greatest for

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[1] Abbreviations: acetylglutamate, *N*-acetyl-L-glutamate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; ATP_A, the ATP molecule that yields P_i in the enzyme reaction; ATP_B, the molecule that provides the phosphate group of carbamoyl phosphate.

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the central complex (enzyme–ADP_A–carboxyphosphate–ATP_B). Recently we have demonstrated that acetylglutamate binds to the 20-kDa, COOH-terminal domain of the rat enzyme (Rodriguez-Aparicio et al., 1989). The *E. coli* enzyme, a heterodimer of two polypeptides (M_r 41 400 and 117 700; Nyunoya & Lusty, 1983; Piette et al., 1984) that are homologous to the NH_2- and COOH-terminal moieties of carbamoyl phosphate synthetase I (Nyunoya et al., 1985), similarly binds a single molecule of each of the activators IMP and ornithine and a single molecule of the inhibitor UMP (Anderson, 1977). These effectors bind to the large subunit (Meister, 1989), and the sites for IMP and UMP overlap (Boettcher & Meister, 1982). The location of the binding sites on the polypeptide is not known. In both the mitochondrial and bacterial carbamoyl phosphate synthetases as well as in the cytosolic enzyme from mammalian liver (carbamoyl phosphate synthetase II, an enzyme involved in pyrimidine synthesis), all of the effectors influence ATP binding and cryoprotectants partly replace the activators (Meister & Powers, 1978; Ishida et al., 1977; Rubio et al., 1983a,b; Rubio & Llorente, 1982). These similarities suggest a common mechanism for the allosteric modulation of enzyme activity, and further, since there are extensive sequence homologies between various carbamoyl phosphate synthetases (Nyunoya et al., 1985; Souciet et al., 1989), they suggest that the effectors bind to a common domain homologous to the COOH-terminal domain involved in acetylglutamate binding. We demonstrate this here for UMP with the *E. coli* enzyme.

Materials and Methods

Chemicals and Enzymes. Carbamoyl phosphate synthetase (specific activity 3.8 units/mg of protein) was isolated from *E. coli* strain RC50/pMC41 (Rubino et al., 1987) and was stored at −70 °C in 2.5 M ammonium sulfate. Immediately before use, it was dissolved in the appropriate buffer and ammonium sulfate was removed by centrifugal gel filtration (Penefsky, 1977) through Sephadex G-25. Elastase (Type IV, porcine pancreas), α-chymotrypsin (Type I-S, bovine pancreas), staphylococcal V8 protease (Type XVII, from *Staphylococcus aureus*), and pyruvate kinase and lactate dehydrogenase (both from rabbit muscle, salt-free) were from Sigma. Trypsin (bovine pancreas) was from Boehringer or, when the incubations included SDS, from Sigma (TPCK-treated). [U-^{14}C]UMP (0.45 Ci/mmol) was from CEA (Centre d'Etudes Nucléaires de Saclay, F-91191 Gif-sur-Yvette Cedex, France).

Assay of Carbamoyl Phosphate Synthetase Activity. The enzyme was assayed spectrophotometrically at 340 nm and 37 °C in 1 mL of a solution at pH 7.6 containing 50 mM glycylglycine, 15 mM $MgSO_4$, 5 mM ATP, 2.5 mM phosphoenolpyruvate, 50 mM $KHCO_3$, 10 mM glutamine [or, when replaced by ammonia, 35 mM $(NH_4)_2SO_4$], 10 mM ornithine, 0.25 mM NADH, 0.04 mg/mL pyruvate kinase, and 0.02 mg/mL lactate dehydrogenase. An enzyme unit synthesizes 1 μmol of carbamoyl phosphate/min, corresponding to a change in absorbance of 12.4 absorbance units/min.

Photoaffinity Labeling. The enzyme (1–2 mg/mL), in a solution of 0.1 M Tris-HCl, pH 7.8, and 0.1 M KCl, was incubated with 9 μM [U-^{14}C]UMP at 22 °C for 15 min. The solution (50–300 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 1 × 0.2 cm. A Mineralight UVGL-58 lamp was held vertically, so that the filter of the lamp was 2 cm from, and parallel to, the face of the cuvette, giving an optical pathlength through the solution of 0.2 cm. The mixture was irradiated at the short wavelength for 3 min, except when indicated. The magnitude of labeling was determined by precipitation with 10% trichloroacetic acid. When the photolabeled enzyme had to be subjected to proteolytic digestion or to formic acid treatement (see below), unbound UMP was removed and the enzyme was suspended in the appropriate incubation buffer by centrifugal gel filtration.

Limited Proteolysis. The synthetase (1–2 mg/mL) was incubated at 37 °C with the indicated protease in the solution specified. Incubations were terminated by dilution in the enzyme assay medium (this prevented further proteolytic inactivation) or, with samples taken for SDS–PAGE, by placing the protein, either by dilution, supplementation with the appropriate components, or centrifugal gel filtration, in sample buffer for electrophoresis (0.125 M Tris-HCl, pH 6.8, 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue) and immediately boiling the sample for 4 min. When trypsin was used, 2 mM PMSF was also added to terminate the digestion. SDS–PAGE was carried out according to Laemmli (1970), with 10% polyacrylamide gel slabs unless otherwise specified. The gels were stained with Coomassie 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).

Sequence Analysis. The large fragment generated by trypsin from the large subunit was separated by SDS–PAGE with a 5% slab gel of 15 × 16 × 0.15 cm with no wells. The electrophoresis was run in a Protean cell (Bio-Rad) cooled by circulation of tap water, at a constant current of 100 mA. The fragment was localized by brief Coomassie Blue staining (Hunkapiller et al., 1983), excised from the gel, electroeluted overnight with the Bio-Rad electroelution apparatus (Model 422; buffer, 0.4% ammonium bicarbonate, 0.1% SDS; current, 8 mA/tube), collected in 2.5 mL, and lyophilized. To remove the SDS, the lyophilizate was treated with cold acetone and centrifuged, and the precipitate was dried under vacuum and used for the analysis (Figure 1A).

The fragment of approximately 25 kDa generated by staphylococcal V8 protease was isolated by HPLC (Figure 1B). The synthetase (1 mg/mL), in a buffer of 0.125 M Tris-HCl, pH 6.8, and 0.5% SDS, was heated for 5 min at 100 °C and cooled, and 0.01 mg/mL V8 protease was added. The solution was incubated for 20 min at 37 °C and then heated for 5 min at 100 °C to inactivate the protease. After being cooled, the protein was precipitated with 9 volumes of acetone at 0 °C, dried under vacuum, dissolved in 70% formic acid, and subjected to HPLC with a Vydac reverse-phase 5-μm C-18 column (Type 218TP54, 0.46 × 25 cm) and a 30-min linear gradient from 20 to 100% acetonitrile (0.1% trifluoroacetic acid present), at a flow rate of 1 mL/min. The optical absorption of the effluent was monitored at 214 nm, and peaks were collected manually. SDS–PAGE demonstrated that the fragment was eluted free from other components as a single peak at 17.2 min (peak b, Figure 1B). The fraction containing this peak was lyophilized and used for sequencing.

The NH_2-terminal sequences were determined at the Servicio de Secuenciación of the University of Barcelona by automated Edman degradation with an Applied BioSystems 470A Protein Sequencer. The phenylthiohydantoins were analyzed on an Applied BioSystems 120A on-line HPLC system using a microbore C-18 Brownlee column.

Cleavage at Aspartyl–Prolyl Bonds. A 78-μL aliquot of a solution of 7 M guanidine hydrochloride containing 1.5

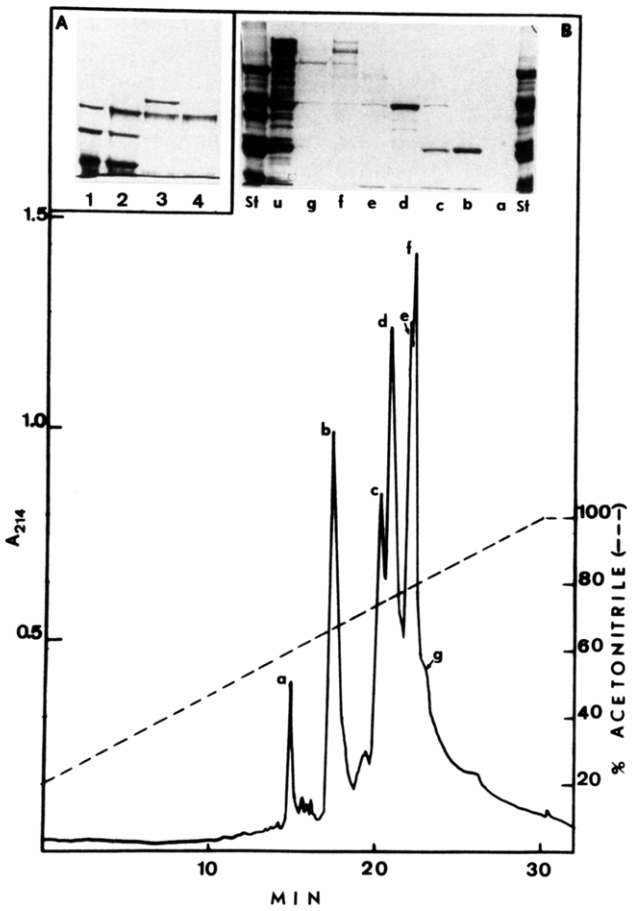

FIGURE 1: Fragments isolated for sequence analysis. (A) SDS–PAGE (5% gel, Coomassie staining) of the large fragment generated by trypsin. Track 1, molecular weight markers of Mr 116 000, 97 400, 66 000 and 45 000 (migrating at the front). Track 2, mixture of the electroeluted fragment and the molecular weight markers. Track 3, mixture of the fragment and intact *E. coli* carbamoyl phosphate synthetase (the large subunit is at the top and the small subunit migrates with the front). Track 4, fragment alone. Note the anomalous migration of the large subunit and the fragment: they exhibit apparent M_r values of 130 000 and 116 000 whereas the molecular weight of the large subunit, deduced from the sequence, is 117 700. (B) Graph: isolation by HPLC of the 25-kDa fragment (peak b) generated by staphylococcal V8 protease. Coomassie-stained SDS–polyacrylamide gel: protein content of the major peaks (tracks a–g) and of the unfractionated mixture (u). St, molecular weight markers (M_r of 66 000, 45 000, 36 000, 29 000, 24 000, 20 100, and 14 200; the last marker migrates at the front).

mg/mL photolabeled enzyme was mixed with 309 μL of 7 M guanidine hydrochloride in formic acid, and the mixture was incubated at 37 °C (Landon, 1977). Samples taken after the indicated periods of incubation were placed in sample electrophoresis buffer by centrifugal gel filtration and were subjected to SDS–PAGE in a 15% polyacrylamide slab gel.

Results

Limited Proteolysis of Carbamoyl Phosphate Synthetase. The results shown in Figure 2 demonstrate that the enzyme is inactivated by incubation with elastase and that ornithine and UMP (main figure) and IMP (inset) protect the enzyme from this inactivation. The inactivation was the same whether carbamoyl phosphate synthetase activity was assayed with glutamine or ammonia as the nitrogen donor (data not shown). Inactivation was associated with the cleavage of the large subunit, yielding a polypeptide approximately 15 kDa smaller (Figure 2). Trypsin and chymotrypsin cleave the enzyme similarly (Figure 2, inset). The fragment of approximately 100 kDa[2] generated with trypsin was isolated by electroelution

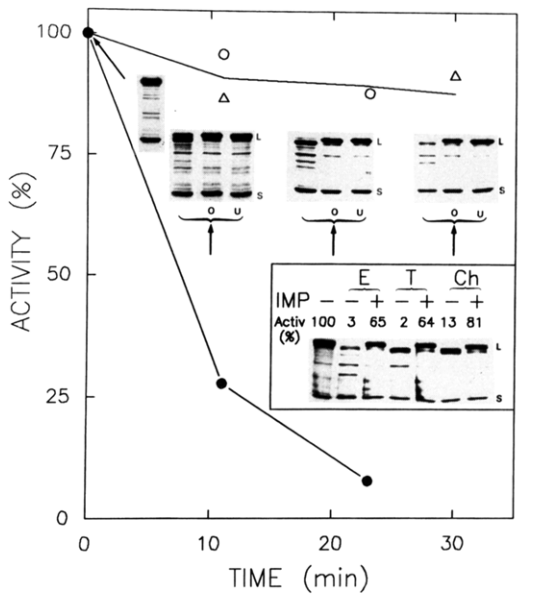

FIGURE 2: Limited proteolysis of carbamoyl phosphate synthetase. The synthetase (1.6 mg/mL) in 95 mM potassium phosphate buffer, pH 7.7, was assayed after the indicated period of incubation at 37 °C with elastase (0.014 mg/mL) in the absence (●) or in the presence of 6 mM ornithine (○) or 3 mM UMP (△). The small amount of UMP (6 μM) carried with the sample into the assay did not cause substantial inhibition. Samples were taken at 10, 20, and 30 min (see arrows) for SDS–PAGE. Gels were stained with Coomassie. L and S denote the large and small subunits of *E. coli* carbamoyl phosphate synthetase. The gel tracks are labeled O (presence of ornithine) and U (presence of UMP) or are unlabeled (absence of either effector). Inset: Influence of IMP (8 mM) on proteolysis of the enzyme. In E, T, and Ch, the synthetase was incubated for 10 min with elastase (24 μg/mL), trypsin (4.6 μg/mL), or chymotrypsin (5 μg/mL). The first lane (unlabeled) illustrates the enzyme incubated without proteases. Enzyme activity, relative to that in the absence of proteases, is shown above the tracks. SDS–PAGE, 7.5% polyacrylamide, Coomassie staining.

from a preparative SDS–polyacrylamide gel and was subjected to 12 cycles of NH_2-terminal sequencing. The sequence, Pro-Lys-Arg-Thr-Asp-Ile-Lys-Ser-Ile-Leu-Ile-Leu, is identical with the NH_2-terminal sequence of the intact large subunit (Nyunoya & Lusty, 1983), demonstrating that the cleavage occurs close to the COOH terminus of the enzyme. Thus, the COOH-terminal region of about 15 kDa is essential for activity and is connected to the remainder of the large subunit by a highly accessible hinge. The accessibility of the hinge is strongly influenced by the presence of allosteric effectors, suggesting that the effectors bind in the vicinity of the hinge and influence its conformation. These findings resemble the observations of Rodriguez-Aparicio et al. (1989) with carbamoyl phosphate synthetase I. The COOH-terminal domain of carbamoyl phosphate synthetase I, which is homologous to the region excised here (Nyunoya et al., 1985), is essential for activity. Binding of acetylglutamate (the allosteric activator of the ureotelic synthetase) accelerates proteolytic cleavage of the hinge joining this domain to the remainder of the enzyme, and thus, acetylglutamate influences the accessibility of this hinge.

Photoaffinity Labeling with [^{14}C]UMP. UMP was found to be suitable for photoaffinity labeling of the enzyme. UMP strongly absorbs UV light, and we found that irradiation under the conditions of the photolabeling experiments (see Materials and Methods) destroyed the nucleotide with a $t_{1/2}$ of 12.5 min

[2] The electrophoretic migration of this fragment and of the large subunit are anomalous (see Figure 1A).

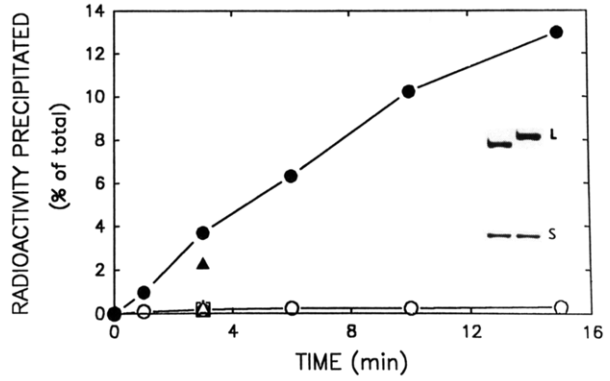

FIGURE 3: Photolabeling of carbamoyl phosphate synthetase by [^{14}C]UMP. Irradiation of mixtures of [^{14}C]UMP (9 μM) and the synthetase (●, ▲, △) or pyruvate kinase (○) (both at 2 mg/mL) was carried out as described under Materials and Methods. In some experiments unlabeled uridine (▲) or UMP (△) at a concentration of 74 μM was added. The experiment denoted by □ shows that pretreatment with trypsin prevents the labeling with [^{14}C]UMP. In this experiment, the synthetase was incubated for 10 min at 37 °C with 10 μg/mL trypsin, in a solution containing 0.1 M Tris-HCl, pH 7.8, and 0.1 M KCl; the digestion was terminated with 2 mM PMSF to stop the digestion; [^{14}C]UMP (9 μM) was then added and the enzyme was irradiated for 3 min. PMSF had no effect on the photolabeling of the enzyme. A sample taken for SDS–PAGE (7.5% polyacrylamide, Coomassie staining) demonstrates complete cleavage by trypsin of the major subunit (see gel within the figure; right and left tracks, enzyme before and after the treatment with trypsin; L and S, large and small subunits).

(assessed by following the fall in the absorbance at 268 nm). Figure 3 demonstrates that upon irradiation of mixtures of the synthetase and [^{14}C]UMP a fraction of the radioactivity was precipitated by trichloroacetic acid. The time course of the labeling was consistent with the kinetics of destruction of UMP by irradiation. Gel filtration through Sephadex G-25 (not shown) and SDS–polyacrylamide gel electrophoresis (Figure 4) confirmed the labeling of the enzyme. The lack of substantial labeling of pyruvate kinase (Figure 3), an enzyme that is not affected by UMP, and the drastic decrease in the labeling of carbamoyl phosphate synthetase upon addition of unlabeled UMP but not of uridine (a poor inhibitor; Pierard, 1966) (Figure 3), indicate that the labeling is specific. In agreement with the well-established fact that the effectors of the enzyme bind to the large subunit (Meister, 1989), only this subunit was labeled (Figure 4), again indicating that the labeling is specific. Cleavage of the 15-kDa COOH-terminal peptide of the large subunit by trypsin treatment abolished the labeling of the enzyme upon subsequent irradiation with [^{14}C]UMP (Figure 3, □ and SDS–PAGE). This result constitutes additional evidence for the specificity of labeling and suggests that UMP is bound within the excised COOH-terminal region.

Photolabeling with [^{14}C]UMP was decreased by the allosteric activators ornithine and IMP (data not shown). The effect of ornithine was restricted to the L isomer and was not observed in the absence of phosphate. In 0.12 M potassium phosphate buffer, pH 7.7, 1 mM L-ornithine inhibited the labeling by 80%. In the same buffer, 1 mM IMP decreased the labeling by 75%, whereas 1 mM inosine (a control) decreased the labeling by only 25% (this decrease was possibly due to absorption of UV light by inosine). These results are consistent with the reported inhibition by ornithine and IMP of UMP binding (Anderson, 1977).

Location of the UMP Site. To minimize photolysis of the enzyme, the period of irradiation used in subsequent experiments was short (3 min), and therefore, only a small fraction (<10%) of the enzyme molecules were labeled. Figure 4 shows

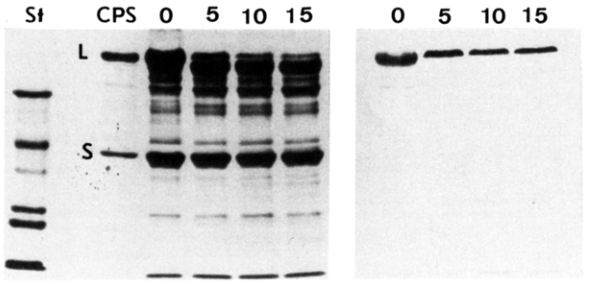

FIGURE 4: Time course of trypsin digestion of carbamoyl phosphate synthetase photolabeled with [^{14}C]UMP. The labeled enzyme (1.5 mg/mL) was incubated at 37 °C with trypsin (1 μg/mL) in 0.12 M sodium phosphate buffer, pH 7.7. Samples were taken for SDS–PAGE after the indicated minutes of incubation. Left, Coomassie staining. Right, fluorography. CPS refers to the nonirradiated enzyme. St refers to molecular weight markers (M_r of 66 000, 45 000, 36 000, 29 000, 24 000, 20 100, and 14 200; the last marker migrates at the front). L and S, large and small subunits of the enzyme.

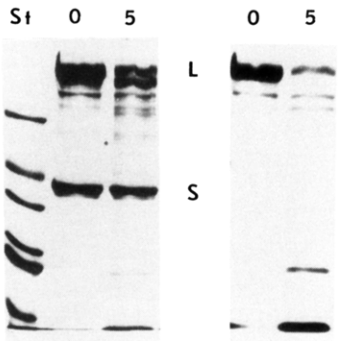

FIGURE 5: Trypsin digestion of the labeled enzyme in the presence of SDS. The photolabeled enzyme (1.2 mg/mL), in a solution of 10 mM Tris-HCl, pH 7.8, 10% glycerol, and 0.5% SDS, was heated for 4 min at 100 °C and cooled to 37 °C, and trypsin (25 μg/mL) was added. Samples taken at the beginning (0 time) and after 5 min of incubation (times indicated above the tracks) were mixed with PMSF (2 mM) and were used for SDS–PAGE. Left, Coomassie staining. Right, fluorography. St refers to molecular weight markers (M_r of 66 000, 45 000, 36 000, 29 000, 24 000, 20 100, and 14 200; the last marker migrates at the front). L and S, positions of the large and small subunits of the enzyme.

that, after irradiation with [^{14}C]UMP and subsequent removal of unbound UMP, the bulk of the enzyme was cleaved by trypsin as described above, but the labeled enzyme molecules were resistant to the attack. This result is to be expected if UMP is cross-linked to its binding site and the resultant covalent complex resembles the normal complex. The labeled enzyme molecules become susceptible to cleavage when the treatment with trypsin is carried out in the presence of 0.5% SDS (Figure 5), indicating that the protection by UMP is due to conformational changes. The pattern of fragmentation is similar to that observed without SDS (Figure 2): the large subunit is cleaved, yielding a fragment approximately 15 kDa smaller, which is not labeled, and the majority of the radioactivity migrates at the front, as expected for a labeled peptide of approximately 15 kDa (Figure 5). These results indicate that UMP binds within the 15-kDa COOH-terminal region of the large subunit that is excised by trypsin. A less conspicuous radioactive band of 25 kDa is also observed (Figure 5), indicating that there is a minor pathway of cleavage of the large subunit in which the hydrolysis takes place at about 25 kDa from the COOH terminus.

We obtained further evidence that [^{14}C]UMP labels at the carboxy terminus by digestion of the enzyme with V8 staphylococcal protease in the presence of SDS (Cleveland et al., 1977). Digestion with V8 protease yielded several fragments

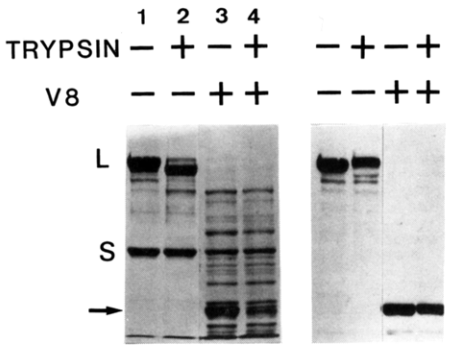

FIGURE 6: Sequential digestion of the labeled enzyme with trypsin in the absence of SDS and with staphylococcal V8 protease in the presence of SDS. Two Eppendorf tubes containing 50 μL of the photolabeled enzyme (2 mg/mL) in 0.12 M sodium phosphate buffer, pH 7.7, were heated for 5 min at 37 °C. Then 2 μL of a solution of 32 μg/mL trypsin was added to one of the tubes, and the incubation was continued for an additional 10 min and was terminated by the addition of 2 mM PMSF and 0.8% SDS (final concentrations), followed by heating at 100 °C for 5 min. After being cooled, the low molecular weight components in the mixtures were replaced by a buffer of 0.125 M Tris-HCl, pH 6.8, 10% glycerol, and 0.1% SDS by using centrifugal gel filtration. A sample of the trypsin-treated (and untreated) synthetase was incubated for 30 min at 37 °C with 20 μg/mL staphylococcal V8 protease; another sample of the trypsin-treated (and untreated) enzyme was similarly incubated without the V8 protease. After incubation, the mixtures were analyzed by SDS–PAGE. Left, Coomassie staining. Right, fluorography. L and S, large and small subunits of the enzyme. The 25-kDa peptide is indicated by the arrow.

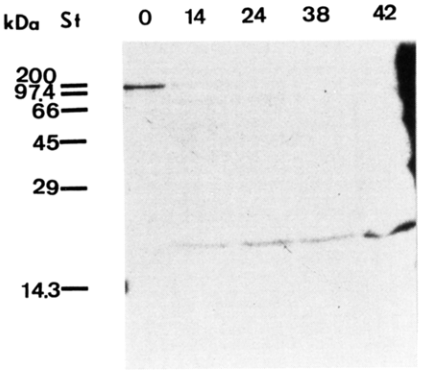

FIGURE 7: Time course of cleavage of photolabeled carbamoyl phosphate synthetase by incubation with 70% formic acid. The number of hours of incubation is indicated above the tracks of the fluorograph (SDS–15% polyacrylamide gel). St refers to ^{14}C-methylated molecular weight markers (from Amersham); their M_r values and exact migration positions are shown. Top radioactive band, large subunit. Bottom radioactive band, fragment of 18.5 kDa.

(Figure 6, left panel, track 3), but only one fragment of 25 kDa (molecular mass determined electrophoretically) contains the label (Figure 6, right panel). Indeed, short-term incubations with low concentrations of V8 protease (5 μg/mL) revealed that the large subunit is first cleaved into complementary fragments of 25 and 105 kDa (molecular masses determined electrophoretically) with the label only in the 25-kDa fragment (results not shown). Treatment with trypsin (in the absence of SDS) before treatment with V8 protease (in the presence of SDS) did not change the fluorographic pattern: all of the radioactivity was still present in the 25-kDa fragment (Figure 6, right panel). This result was expected, since in the absence of SDS, trypsin cleaves only the labeled enzyme molecules. However, Coomassie staining revealed that the 25-kDa band was nearly absent when the enzyme was pretreated with trypsin (Figure 6, left panel, track 4). This was the only noticeable change introduced by pretreatment with trypsin. Since trypsin pretreatment removes a 15-kDa fragment from the COOH terminus of unlabeled enzyme molecules (the bulk of the enzyme) (Figure 6, left panel, track 2), the 25-kDa fragment released by V8 protease includes the trypsin cleavage product, and therefore, it is also carboxy-terminal.

That the 25-kDa fragment cleaved by V8 protease is derived from the COOH terminus of the large subunit was confirmed by NH_2-terminal sequencing of the isolated fragment (see Materials and Methods and Figure 1). The sequence found, Val-Tyr-Leu-Ile-Glu-Val-Asn, is identical with the sequence starting at residue 836 of the large subunit (Nyunoya & Lusty, 1983), and is preceded, as expected from the specificity of the V8 protease (Drapeau, 1977), by a glutamate residue. The molecular weight deduced from the published sequence for the peptide starting at residue 836 and ending at the COOH terminus of the subunit is 25 797, in excellent agreement with the M_r value of the fragment determined by SDS–PAGE.

Several nucleotide-binding sequences have been proposed in the large subunit of *E. coli* carbamoyl phosphate synthetase (Nyunoya & Lusty, 1983; Powers-Lee & Corina, 1987). It is conceivable that some of these are involved in the binding of UMP. However, only the most distal sequence (amino acids 844–885) is within the COOH-terminal fragment released by V8 protease and none is within the 15-kDa COOH-terminal peptide released by trypsin. To confirm further that UMP is not cross-linked to the distal nucleotide-binding sequence (residues 844–885), the enzyme was treated with 70% formic acid in the pesence of guanidine hydrochloride to cleave specifically aspartyl–prolyl bonds (Landon, 1977). Four such peptide bonds exist in the large subunit of *E. coli* carbamoyl phosphate synthetase, at positions 56-57, 409-410, 416-417, and 903-904 (Nyunoya & Lusty, 1983). Cleavage of the four bonds would give five fragments of 6, 38.4, 0.8, 54.1 and 18.5 kDa, ordered from the NH_2 end of the subunit. The distal nucleotide-binding sequence is located in the 54.1-kDa fragment. However, SDS–PAGE and fluorography after incubation of the labeled enzyme with formic acid demonstrate that the radioactivity accumulates only in a single band of M_r 18 500 (Figure 7). Coomassie staining (not shown) also revealed bands migrating at positions consistent with M_r values of 38 000 and 54 000 that were not radioactive. In addition, no radioactivity was observed at the dye (bromphenol blue) front, where peptides of 6 and 0.8 kDa would migrate (Figure 7). These results are to be expected if the enzyme is labeled exclusively within the COOH-terminal 15 kDa of the large subunit. Thus, the involvement of any of the previously proposed nucleotide binding sequences in the binding of UMP is excluded.

Discussion

In the early stages of this study we synthesized 5-azido-UMP from UMP (Evans & Haley, 1987), and we found that this highly photoreactive analogue inhibits the enzyme in a manner similar to UMP (data not shown). TMP is also a good inhibitor (Boettcher & Meister, 1982), indicating that substituents at C-5 of the pyrimidine ring do not influence the binding. In contrast, substituents at C-4 are important, for CMP is not an inhibitor (Boettcher & Meister, 1982).

We also synthesized 5-azido-[^{14}C]UMP and showed that it photolabels the enzyme specifically (as demonstrated in Figure 3) and rapidly (data not shown). The labeled enzyme was somewhat less protected from proteolysis than the enzyme labeled with UMP, revealing subtle differences in the conformation of the enzyme cross-linked to UMP and to 5-azido-UMP (that the protection is due to conformational factors is shown by its abolition by SDS; see Results). The protection

is likely to be due to the phosphoribose moiety of UMP and 5-azido-UMP rather than to the pyrimidine ring, since the photolysis destroys this ring but does not destroy the phosphoribose moiety (Evans & Haley, 1987), which thus is important in the interaction with the enzyme. This is consistent with earlier findings that dUMP and uridine are poor inhibitors and that araUMP is an activator (Pierard, 1966; Boettcher & Meister, 1982).

The present studies have also provided information on the domain structure of the large subunit of *E. coli* carbamoyl phosphate synthetase. Since the amino acid sequences of this subunit and of the equivalent region of the rat liver enzyme are highly conserved (41% identities, 20% conservative replacements) (Nyunoya et al., 1985), the two proteins are likely to have similar structural domains. Rat and hamster carbamoyl phosphate synthetase I have been shown to be composed, starting from the N-terminus, of four structural domains of about 40, 40, 60, and 20 kDa, of which the NH_2-terminal domain of 40 kDa is homologous to the small subunit of the bacterial enzyme (Powers-Lee & Corina, 1986, 1987; Evans & Balon, 1988; Marshall & Fahien, 1988). Using several proteases, we demonstrate here that the COOH-terminal domain in the large subunit of the *E. coli* enzyme is analogous to the 20-kDa COOH-terminal domain of the rat liver enzyme: this domain is cleaved first,[3] cleavage results in inactivation, and the susceptibility to cleavage is strongly influenced by the presence of allosteric effectors. In addition, trypsin and elastase (but not chymotrypsin) further cleave the truncated large subunit, yielding a fragment approximately 40 kDa smaller (Figure 2). This fragment probably corresponds to the 60-kDa domain of the rat liver enzyme (Powers-Lee & Corina, 1986; Evans & Balon, 1988; Marshall & Fahien, 1988).

We show here that UMP binds specifically and labels the protein within 15 kDa (about 140 amino acids) of the COOH terminus of the large subunit. The binding of UMP to the COOH-terminal domain of the *E. coli* enzyme and the binding of acetylglutamate to the analogous domain in the rat liver enzyme supports our proposal (Rodriguez-Aparicio et al., 1989) that the COOH-terminal domain of carbamoyl phosphate synthetases is concerned with allosteric control and effector binding and thus is the regulatory domain.[4] Allosteric transitions are known to alter the relationship between different domains in proteins [see, for example, Barford and Johnson (1989) and Schirmer and Evans (1990)]; these alterations may be reflected here in the changes in proteolytic susceptibility induced by the binding of UMP, IMP, and ornithine. Since IMP and ornithine influence the susceptibility to proteolytic attack in a manner similar to UMP, ornithine and IMP may bind also to the COOH-terminal domain. This possibility would be consistent with the inhibition by ornithine and IMP of the labeling by UMP. In fact, observations of Boettcher and Meister (1982) on the effect of UMP analogues strongly suggest that the sites for IMP and UMP overlap. The chemical similarities and the metabolic relatedness of ornithine and acetylglutamate (Davis, 1986; Morris et al., 1969) suggest that ornithine and acetylglutamate bind to topographically equivalent sites in the *E. coli* and rat liver enzymes, respectively.

The COOH-terminal domain of carbamoyl phosphate synthetase from *E. coli* exhibits some intriguing similarities to the regulatory chain of aspartate transcarbamylase from the same microorganism. The regulatory chain has a similar mass (17 kDa), and purine and pyrimidine nucleotides (ATP and CTP) bind at overlapping sites, ATP being an activator and CTP an inhibitor (Kantrowitz & Lipscomb, 1988; Allewell, 1989). Moreover, UTP is an inhibitor in the presence of CTP (Wild et al., 1989), and UTP may also bind to the regulatory chain. Sequence comparison (Queen & Korn, 1984) between the terminal domain of carbamoyl phosphate synthetase (Nyunoya & Lusty, 1983) and the regulatory chain of aspartate transcarbamylase (Kim et al., 1987) (both from *E. coli*) does not reveal a strong conservation (for residues 900–1031 of carbamoyl phosphate synthetase, 24.8% identities, 15.6% conservative replacements, and 5 gaps/100 residues). In comparison, the COOH-terminal domain of the synthetase compared with the homologous region of the NH_2 half of the enzyme (Nyunoya et al., 1985) yields 25% identities, 15.5% conservative replacements, and 5.4 gaps/100 residues; and the COOH-terminal domain of the *E. coli* enzyme compared with the homologous region of the rat (Nyunoya et al., 1985) and yeast (Lusty et al., 1983) enzymes yields, respectively, 27.4% and 27.8% identities, 15.9% and 13.1% conservative replacements, and 3.3 and 4.9 gaps/100 residues. The latter comparisons indicate that the amino acid sequence of the COOH-terminal domain has undergone extensive variation. Thus, the regulatory chain of aspartate transcarbamylase and the regulatory domain of carbamoyl phosphate synthetase might be evolutionarily related.[5] Crystallographic data on the tertiary structure of the enzyme would be essential to test further this possible relation.

Previous studies of the rat liver enzyme led us to propose (Rodriguez-Aparicio et al., 1989) that the regulatory domain is involved in facilitating the binding of ATP_A (the molecule of ATP that yields phosphate) and in closure of the binding site to allow formation of carboxyphosphate in the absence of water. Consistent with this view, cleavage of the COOH-terminal domain of carbamoyl phosphate synthetase results in complete loss of activity[6] (Powers-Lee & Corina, 1986; Evans & Balon, 1988; Marshall & Fahien, 1988; present data). Post et al. (1990) have shown that the NH_2 half of the large subunit is involved in the ATPase partial reaction (which takes place at the ATP_A site) and the COOH half of the subunit is involved in the partial reaction of the ATP synthesis (which takes place at the ATP_B site). Thus, the allosteric domain is adjacent, in sequence, to the domain involved in the reaction of ATP_B rather than ATP_A. Nonetheless, the two halves of the large subunit (present results) or of the homologous regions of carbamoyl phosphate synthetase I (Powers-Lee & Corina, 1986; Evans & Balon, 1988; Marshall & Fahien, 1988; our unpublished results) fold into corresponding domains. Thus, the large subunit can be considered a homodimer, rendering highly likely a complementary isologous association (Figure 8) (Klotz et al., 1975) between its two halves.[7] Such an

[3] The fact that trypsin and V8 staphylococcal protease cleave the COOH-terminal domain even after the synthetase has been boiled in SDS indicates that this treatment does not abolish the tertiary structure of the enzyme or that the enzyme refolds (at least partly) on cooling.

[4] The phosphorylation of the multifunctional polypeptide CAD at the COOH-terminal region of the carbamoyl phosphate synthetase II component, with concomitant loss of the inhibitory effect of UTP (Carrey & Hardie, 1988), supports the existence of a COOH-terminal regulatory domain in carbamoyl phosphate synthetase II and suggests that UTP binds in this domain.

[5] The gene coding for the large subunit of carbamoyl phosphate synthetase may have arisen from fusion (or recombination) of an ancestral gene for carbamate kinase (which is homologous to the initial 300 amino acids of the COOH half; Baur et al., 1989) and the gene(s) encoding the distal putative nucleotide site (Nyunoya et al., 1985) and the allosteric domain, followed by duplication.

[6] A close association between the allosteric domain and an ATP site is supported by the labeling of the COOH-terminal domain by [^{14}C]-5′-(fluorosulfonylbenzoyl)adenosine (Potter & Powers-Lee, 1990).

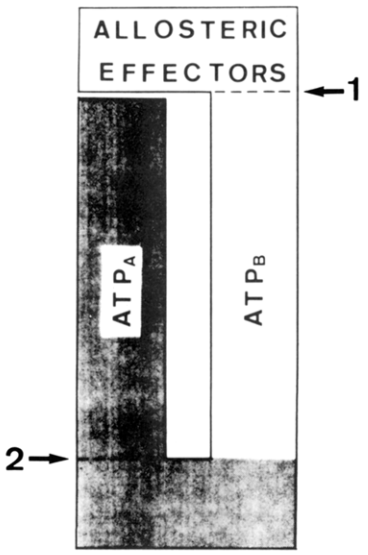

FIGURE 8: Schematic representation of how isologous dimerization of the large subunit of carbamoyl phosphate synthetase (or the equivalent part of the chain of those enzymes that do not have a separate small subunit) brings the nucleotide site involved in the reactions of ATP_A close to the COOH-terminal domain of the enzyme that we have demonstrated to bind effectors. The polypeptide chain is a tandem repeat (Nyunoya et al., 1983). The NH_2-terminal half of the chain is shadowed. ATP_A and ATP_B indicate the domains involved in the reactions of ATP_A and ATP_B (Post et al., 1990). "Allosteric Effectors" indicates the COOH-terminal domain that binds the effectors of the enzyme. The arrow labeled 1 represents the junction between the COOH-terminal domain and the rest of the enzyme. Proteolysis occurs first at this site. The arrow labeled 2 represents the corresponding junction on the NH_2-terminal segment. Proteolysis at this site follows proteolysis at site 1, consistent with interactions between the COOH-terminal and the NH_2-terminal domains. The COOH-terminal domain appears to control access to the ATP_A site. The small subunit is not represented, but it is known to interact with the two ATP-binding domains (Guillou et al., 1989). For further details, see text.

association would place the COOH-terminal domain of the large subunit close to the NH_2 terminus and thus to the ATP_A binding site (Figure 8), as our previous data had demanded (Rodriguez-Aparicio et al., 1989). In such a dimer both sites for ATP would be juxtaposed, consistent with earlier studies showing that the *E. coli* enzyme is inhibited by Ap_5A (Meister & Powers, 1978). Thus, the catalytic reaction could take place at the interface between the domains for the ATP_A and ATP_B, thus favoring the sequential attack on the carbon by the terminal phosphoryl groups of the two nucleotides (Meister, 1989). Channeling of ammonia to the carboxyphosphate intermediate requires interaction of the small subunit with the catalytic center. The NH_2-terminal moiety of the small subunit has been shown to interact with the NH_2-terminal regions of both halves of the large subunit (Guillou et al., 1989). In the homodimer proposed here, both these regions would be close together and the small subunit bound to this region would be appropriately placed to deliver ammonia to the catalytic center.

Acknowledgments

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[7] Limited proteolysis of the rat liver enzyme and gel filtration of the fragments support this view: complexes of the cleaved second and third domains (counting from the NH_2 terminus) were detected (Guadalajara, 1987).

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Structure and Expression of the Bovine Amelogenin Gene[†,‡]

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ABSTRACT: In order to define further the mechanisms responsible for tooth amelogenin heterogeneity, seven bovine amelogenin cDNAs were sequenced. On the basis of these sequences, five of the cDNAs could be grouped into one class which differed appreciably in sequence from the second group of two cDNAs. Two overlapping bovine genomic clones were then isolated and shown by sequencing to contain six exons encoding the entire consensus sequence of the class I cDNA. Southern blot analysis of DNA from male and female animals using class I or class II specific oligonucleotide probes suggested that the class I gene sequence was located on the X chromosome while the class II sequence was located on the Y chromosome. Therefore, these results also suggest that the genes on the X and Y chromosomes are both transcribed. Furthermore, the results are consistent with alternative splicing of the class I primary transcript as a potential mechanism for generating amelogenin heterogeneity.

During enamel formation by ameloblasts, the extracellular matrix that is first secreted is relatively high in protein content (30%) but declines during the maturation phase to less than 2% (Frank, 1979; Burgess & McClaren, 1965). Although these proteins appear to be a complex mixture containing up to 20 components, it has been hypothesized that they are derived from a relatively few high molecular weight precursors by programmed degradation or artifactual degradation during sample preparation (Robinson et al., 1982; Sasaki et al., 1982). The proteins obtained from mammalian enamel have been divided operationally into two classes, amelogenins and enamelins, based upon their solubilization with powerful chaotropic solvents such as guanidine hydrochloride (Gu·HCl). Amelogenins are solubilized by extraction with 4 M Gu·HCl alone, while 4 M Gu·HCl containing 0.5 M EDTA to dissolve the mineral phase is required to solubilize the enamelins (Termine et al., 1980). The majority of the protein in the amelogenin fraction is found in the molecular weight range of 10K–30K, although small amounts of higher molecular weight components (~40K) have been observed (Belcourt et al., 1983; Christner et al., 1985). In contrast, the majority of the protein in the enamelin fraction has an apparent molecular weight of approximately 70K (Termine et al., 1980; Christner et al., 1985). These two classes of proteins also differ appreciably in amino acid composition (Termine et al., 1980). Furthermore, monoclonal antibodies have been obtained which appear to be specific either for amelogenin or for enamelin, but do not cross-react (Christner et al., 1985; Rosenbloom et al., 1986). Taken together, these lines of evidence support the concept that amelogenins and enamelins are distinct classes of proteins.

Mouse (Snead et al., 1985) and bovine (Shimokawa et al., 1987a,b) amelogenin cDNAs have been cloned, and the deduced amino acid sequences agree well, but not precisely, with those previously obtained by extensive protein sequencing of

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