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Organization of a multifunctional protein in pyrimidine biosynthesis

A domain hypersensitive to proteolysis

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When the multifunctional protein that catalyses the first three steps of pyrimidine biosynthesis in hamster cells is treated with staphylococcal V8 proteinase, a single cleavage takes place. The activities of carbamoyl-phosphate synthetase (EC 6.3.5.5), aspartate carbamoyltransferase (EC 2.1.3.2) and dihydro-orotase (EC 3.5.2.3) and the allosteric inhibition by UTP are unaffected. One fragment, of M_r 182000, has the first and third enzyme activities, whereas the other fragment, of M_r 42000, has aspartate carbamoyltransferase activity and an aggregation site. A similar small fragment is observed in protein digested with low concentrations of trypsin. A similar large fragment is seen after digestion with trypsin and as the predominating form of this protein in certain mutants defective in pyrimidine biosynthesis. These results indicate that a region located adjacent to the aspartate carbamoyltransferase domain is hypersensitive to proteinase action *in vitro* and may also be sensitive to proteolysis *in vivo*.

In mammals, the first three enzymes in pyrimidine biosynthesis (carbamoyl-phosphate synthetase, EC 6.3.5.5; aspartate carbamoyltransferase, EC 2.1.3.2; and dihydro-orotase, EC 3.5.2.3) are covalently linked on a single multifunctional polypeptide of M_r about 220000 (Mori & Tatibana, 1975; Coleman *et al.*, 1977; Jarry, 1978; Davidson & Patterson, 1979). The native form of this protein, known as 'CAD,' is a multimeric aggregate of identical subunits (Coleman *et al.*, 1977; Jarry, 1978; Mori & Tatibana, 1978). In preparations of CAD protein from a CAD-overproducing Syrian-hamster cell line (Coleman *et al.*, 1977) and from wild-type Chinese-hamster ovary cell line K1 (Davidson & Patterson, 1979), low concentrations of a nicked form (about 200kDa in size) of this protein have been observed. A similar nicked form has been found to predominate in some mutants of Chinese-hamster ovary K1 cells that are defective in pyrimidine biosynthesis (Davidson & Patterson, 1979). Several of these mutants have high levels of carbamoyl-phosphate synthetase and dihydro-orotase (50% of wild-type) and low, but detectable, levels of aspartate carbamoyltransferase (Patterson & Carnright, 1977; Davidson *et al.*, 1979). Only the first and third enzymes appear to be linked in the nicked form of CAD, whereas the second enzyme is unlinked (Davidson *et al.*, 1979).

In view of these results, it was hypothesized that (i) a proteinase-susceptible region exists in the normal CAD protein which leads to the release of the aspartate carbamoyltransferase domain, and (ii) this region appears to become even more sensitive to proteolytic cleavage in the mutants (Davidson & Patterson, 1979). However, recent experiments involving the treatment *in vitro* of normal CAD with proteinases demonstrated the release of several fragments (Davidson *et al.*, 1981; Mally *et al.*, 1981).

For example, treatment of CAD with high concentrations of trypsin yields three fragments: a 129000-M_r fragment with carbamoyl-phosphate synthetase activity; a 39000-M_r fragment with

Abbreviations used: CAD, the multifunctional protein carrying the first three enzymes of pyrimidine biosynthesis; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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aspartate carbamoyltransferase activity; and a 44000-M_r fragment with dihydro-orotase activity (Davidson *et al.*, 1981). The data obtained *in vitro*, therefore, are not consistent with the findings *in vivo*.

In an attempt to resolve this apparent discrepancy, experiments were undertaken using a proteinase with a different specificity and also lower concentrations of trypsin. The present paper describes the fragments produced when CAD protein is treated *in vitro* with dilute concentrations of trypsin and staphylococcal V8 proteinase. Evidence is presented which is consistent with the data obtained *in vivo*, establishing that a region of hypersensitivity to proteolysis does exist between the aspartate carbamoyltransferase domain and the rest of the protein.

Material and methods

Cells

CAD protein was purified (Davidson *et al.*, 1981) from Syrian-hamster cell line 165/28, which overproduces this protein 50–100-fold (Coleman *et al.*, 1977; Kempe *et al.*, 1976). LN45 is a Chinese-hamster cell line derived by nitrogen mustard ICR 191 mutagenesis from Urd$^-$A, a strain which requires uridine for growth and is defective in the CAD protein (Patterson & Carnright, 1977). LN45 can grow in the absence of uridine and is similar to the mutants B48 and D20 described elsewhere (Davidson & Patterson, 1979; Davidson *et al.*, 1979). This mutant has about 50% of the wild-type activity of carbamoyl-phosphate synthetase and dihydro-orotase and about 1–3% of the wild-type activity of aspartate carbamoyltransferase. LN45 was preferred in these studies because it has a high level of altered CAD protein. CAD protein was isolated from this mutant by using the immunoprecipitation technique described previously (Davidson & Patterson, 1979).

Proteinase treatment

Purified CAD protein was digested for 30 min at 37°C with diphenylcarbamoyl chloride-treated trypsin (Sigma) or staphylococcal proteinase V8 (Miles) (Davidson *et al.*, 1981). Digestion was terminated by placing the reaction mixture on ice.

Polyacrylamide-gel electrophoresis

Non-denaturing gel electrophoresis was performed as previously described (Davidson *et al.*, 1981), except that the polymerized gel was soaked for 24h in buffer containing 30% (v/v) dimethyl sulphoxide and 5% (v/v) glycerol. SDS/polyacrylamide-slab-gel electrophoresis was performed as described by Laemmli (1970; see also Davidson & Patterson, 1979) with a 5–20% (w/v) linear gradient of polyacrylamide except where noted.

Two-dimensional electrophoresis was performed as follows. Staphylococcal-V8-proteinase-digested CAD protein was separated by non-denaturing gel electrophoresis. The gel was stained, destained, and equilibrated with SDS/gel-electrophoresis buffer as described by Stralfors & Belfrage (1983). Stained bands were excised from the slab gel, boiled in SDS sample buffer (Laemmli, 1970) for 3 min, and loaded into wells of an SDS-containing slab gel. Melted agarose gel (1%, w/v) was layered over the gel slices. Electrophoresis was then performed as described by Laemmli (1970).

Assays

Carbamoyl-phosphate synthetase, aspartate carbamoyltransferase and dihydro-orotase were assayed in gels or aqueous samples as described previously (Patterson & Carnright, 1977; Davidson *et al.*, 1981). Inhibition of carbamoyl-phosphate synthetase by UTP was measured in aqueous samples in a 200 μl reaction mixture containing 50 mM-Hepes, pH 7.4, 50 mM-KCl, 50 mM-$NaHCO_3$, 1 mM-ATP, 3 mM-$MgCl_2$, and 12 mM-L-[^{14}C]glutamine (248 mCi/mmol; New England Nuclear Corp.) in the presence or absence of 2 mM-UTP. The assay mixture was incubated and treated as described in the standard assay for carbamoyl-phosphate synthetase (Davidson *et al.*, 1981).

Concentrations of CAD protein and proteinases were quantified by the Coomassie Blue method (Bradford, 1976; Spector, 1978; Read & Northcote, 1981), with Bio-Rad reagents and bovine serum albumin as protein standard.

Results

Digestion with staphylococcal V8 proteinase

Purified CAD protein was digested with various concentrations of V8 proteinase and the protein separated by SDS/polyacrylamide-gel electrophoresis. As Fig. 1 shows, two major products result with M_r values of 182000 and 42000. Over a broad range of proteinase concentrations, activities of carbamoyl-phosphate synthetase, aspartate carbamoyltransferase and dihydro-orotase remain high, even after more than 90% of the full-length CAD protein has been cleaved (Table 1). Inhibition by UTP of the carbamoyl-phosphate synthetase activity also remains high after cleavage of the CAD protein with V8 proteinase (Table 2). The mutant D20, in which the nicked form of CAD predominates (Davidson & Patterson, 1979), also shows near-normal allosteric regulation by both

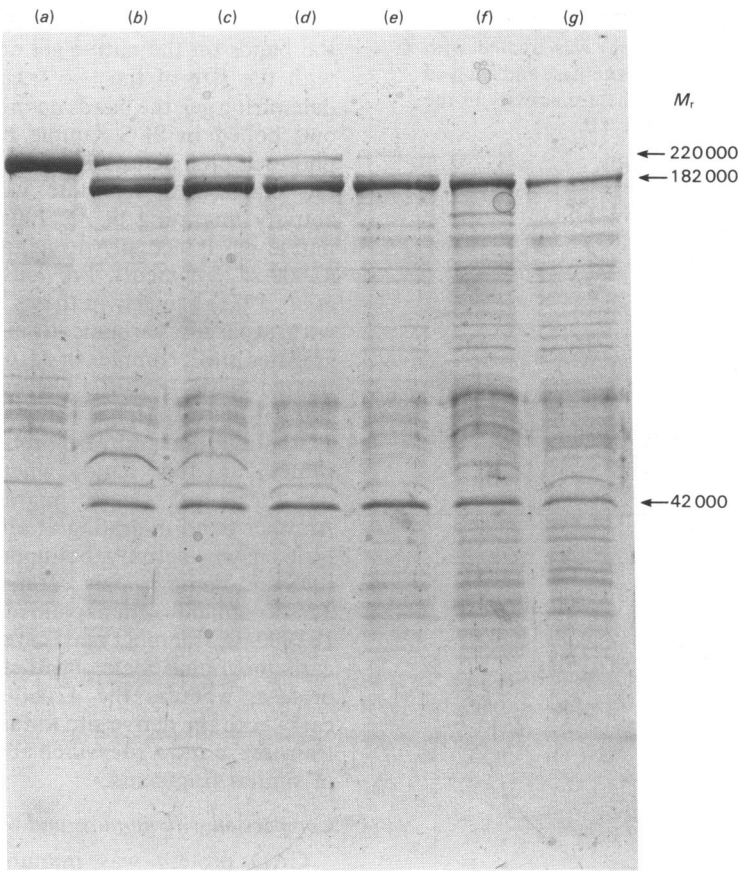

Fig. 1. *Digestion of CAD protein with staphylococcal V8 proteinase*
Purified CAD protein (2.8 μg in 10 μl) was digested with various concentrations of V8 proteinase for 30 min at 37°C. Protein was denatured by boiling for 3 min in 3% SDS and 0.7 M-β-mercaptoethanol and was separated by SDS/polyacrylamide-gel electrophoresis. Final concentrations of V8 proteinase (μg/10 μl): 0 (*a*), 0.01 (*b*), 0.02 (*c*), 0.04 (*d*), 0.08 (*e*), 0.16 (*f*), 0.32 (*g*).

Table 1. *Stability of enzyme activities and protein after proteinase treatment*
After treating purified CAD protein (10 μg) with various concentrations of V8 proteinase for 30 min at 37°C, each of the first three enzymes of pyrimidine biosynthesis were assayed and compared with those of undigested protein. Samples were denatured, electrophoresed in the presence of SDS, and stained. The absorbance of the full-length protein was determined and compared with that of an undigested sample.

	Activity (% of initial)			
Proteinase (μg)	Carbamoyl-phosphate synthetase	Aspartate carbamoyl-transferase	Dihydro-orotase	Percentage of full-length CAD
0	100	100	100	100
0.032	102	90	82	18
0.08	144	71	96	6
0.16	118	58	79	5
0.4	103	40	101	1
0.8	89	43	71	0

UTP and 5-phosphoribosyl 1-pyrophosphate (P. C. Rumsby, unpublished work).

In order to relate the two proteolytic fragments to enzyme activities, proteinase-treated CAD protein was electrophoresed under native conditions and the gel sliced and assayed. At M_r

Table 2. *Allosteric regulation of digested CAD*
Purified CAD protein in (10 μg) was treated with various concentrations of V8 proteinase and assayed for carbamoyl-phosphate synthetase activity in the presence or absence of 2mM-UTP.

Proteinase (μg)	Enzyme activity* −UTP	Enzyme activity* +UTP	Inhibition (%)
0	5470	833	84.8
0.038	2752	111	96.0
0.075	3627	78	97.8
0.15	3795	182	95.2
0.3	3021	73	97.6
0.6	3043	131	95.7
1.2	1516	246	83.3

* Radioactivity (c.p.m.) − background radioactivity of 0.1 vol. of total reaction mixture.

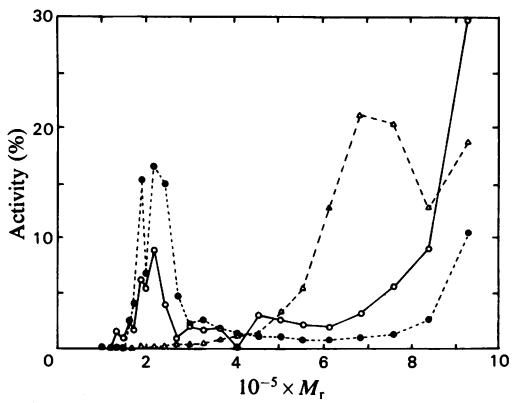

Fig. 2. *Migration of proteinase-treated CAD through native gel*
CAD protein (22.5 μg) was treated with 0.9 μg of V8 proteinase in a total volume of 150 μl for 30 min at 37°C. Digest was applied to three wells of a native polyacrylamide gel (gradient 3.5–20%) and electrophoresis performed for 18 h at 4°C. Gel lanes were cut into 0.25 cm sections and assayed for carbamoyl-phosphate synthetase (○), aspartate carbamoyltransferase (△), and dihydro-orotase (●). Thyroglobulin (669000), ferritin (440000), catalase (232000), lactate dehydrogenase (140000) and albumin (67000) were used as M_r standards.

greater than 800000 all three enzyme activities co-migrate and is likely to represent uncleaved CAD protein (Davidson *et al.*, 1981). At M_r less than 800000, the carbamoyl-phosphate synthetase and dihydro-orotase activities co-migrate through the gel, whereas the aspartate carbamoyltransferase activity migrates much higher in the gel (Fig. 2). When the native gel is stained for protein, bands are visible in the regions where enzyme activities were observed (Fig. 3*a*). Because the M_r values of the bands on the native gel do not correlate well with the size of the two fragments seen on the denaturing gel, the bands on the native gel were cut out, boiled in SDS sample buffer, and electrophoresed on a denaturing gel. As Fig. 3(*b*) shows, the band with aspartate carbamoyltransferase activity migrating at M_r 700000–740000 on the native gel is actually an aggregate consisting of 42000-M_r fragments. Previous studies (Davidson *et al.*, 1981) had shown that a 39000-M_r fragment with aspartate carbamoyltransferase activity aggregates into a complex of M_r 660000 after CAD is treated with trypsin. The native gel band at 170000–300000 M_r has both carbamoyl-phosphate synthetase and dihydro-orotase activities. The protein in this band, when separated on a denaturing gel, clearly migrates at M_r 182000. Another band migrating at about 440000 M_r has little enzyme activity, but upon denaturation with SDS and boiling yields a fragment of 182000 M_r. This fragment is unexplained. Nevertheless, the 182000-M_r fragment must carry the active sites for carbamoyl-phosphate synthetase and dihydro-orotase, whereas the 42000-M_r fragment must carry both the active site for aspartate carbamoyltransferase and a site which allows the aggregation of similar fragments.

Comparison with mutants and with trypsin treatment

CAD protein was immunoprecipitated from LN45 and electrophoresed on SDS/polyacrylamide gels with V8-proteinase-digested CAD in order to see if the large fragments of each were similar. A lower-percentage-polyacrylamide gel was used in order to increase separation of the large fragments. Fig. 4(*a*) demonstrates that the two forms, although close in size, can be separated and that the LN45 protein is slightly larger (193000 M_r). A similar comparison was made between CAD protein digested with V8 proteinase and CAD protein digested with a low concentration of trypsin (Fig. 4*b*). The large fragment produced by these digestions do not separate well. However, the tryptic fragment is somewhat larger (185000–195000 M_r). The diffuse band is observed consistently after trypsin treatment and may mean that there are several susceptible sites in close proximity. When a higher-percentage-polyacrylamide gel is used, the small fragments seen after V8 proteinase or trypsin treatment show the complementing difference; i.e. the trypsin fragment is about 3000 M_r smaller than the V8 proteinase fragment. For the protein in LN45, and for CAD protein treatment with low concentrations of trypsin, carbamoyl-phosphate synthetase and dihydro-orotase activities are associated with the large fragment (results not shown).

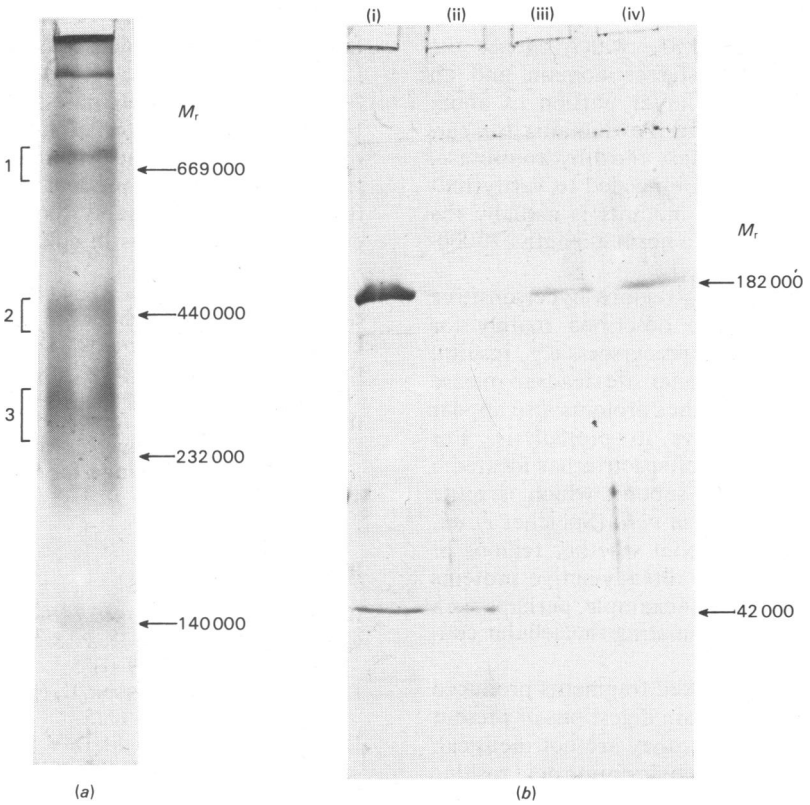

Fig. 3. *Subunit structure of digested fragments*
(*a*) Purified CAD protein (17.8 μg) was digested with V8 proteinase (0.14 μg) as described in Fig. 2. The gel was stained with Coomassie Blue. Aspartate carbamoyltransferase activity correlates with band 1. Carbamoyl-phosphate synthetase and dihydro-orotase correlates with band 3. (*b*) Stained bands from native gel were boiled in SDS sample buffer for 5 min and applied to wells of an SDS/polyacrylamide gel. V8 proteinase (0.05 μg)-treated CAD protein (3.2 μg) as a control (i); bands from native gel: band 1 (ii), band 2 (iii) and band 3 (iv).

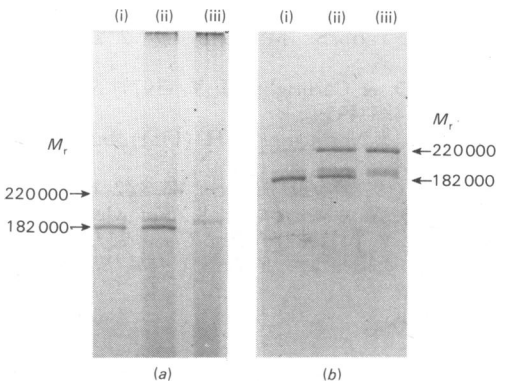

Fig. 4. *Comparison of large fragment of CAD protein*
(*a*) Purified CAD protein (2.8 μg) digested with V8 proteinase (0.02 μg) and CAD protein immunoprecipitated from mutant hamster cell LN45 (2×10^6 cells equivalent) were separated by electrophoresis on an SDS/polyacrylamide gel (4–10% gradient): V8-proteinase-digested CAD only (i); mixture of digested CAD and LN45 CAD (ii); CAD from LN45 only (iii). (*b*) Purified CAD protein (2.8 μg) digested with either proteinase V8 (0.02 μg) or trypsin (0.002 μg) was separated by electrophoresis on an SDS/polyacrylamide gel (4–12% gradient): V8-proteinase-treated CAD only (i); mixture of V8-proteinase- and trypsin-treated CAD (ii); trypsin-treated CAD only (iii). On this type of gel the smaller fragments (< 50000 M_r) run off the bottom of the gel.

Discussion

These experiments demonstrate that treatment of normal CAD protein *in vitro* with low concentrations of trypsin or V8 proteinase can yield a large, nicked, form of CAD similar in size and properties to the nicked form seen in CHO-K1 cells and mutants defective in pyrimidine biosynthesis. Hence, the CAD protein appears to have a region hypersensitive to proteolysis, a region which may be even more sensitive in mutants such as D20 (Davidson & Patterson, 1979) and LN45. This

hypersensitive region exists between a smaller portion of about 42000 M_r, which carries the aspartate carbamoyltransferase domain and an aggregation site, and a larger portion of about 182000 M_r, which carries the domains for carbamoyl-phosphate synthetase and dihydro-orotase. Additional experiments are needed to verify that the CAD protein in the mutants is actually the result of rapid turnover of a normal-length 220000-M_r precursor.

Polypeptides containing regions hypersensitive to proteolysis have been described mainly for hormone and proteinase precursors, e.g. insulin, where proteolysis at particular sites leads to release of the active hormone. Other proteins also appear to have regions sensitive to proteolysis. For example, recent analysis of spectrin has located a protein domain in the β-subunit which is quite susceptible to proteolysis *in vitro* (Speicher *et al.*, 1982). Little is known about whether regions of preferential proteolysis in already-active proteins might serve a function. For example, perhaps such sites play a role in self-regulating the cellular concentrations of proteins.

Although the larger nicked fragments produced by V8 proteinase and trypsin digestions or present in cells are similar in size, they are not identical. This shows that it is not a single amino acid residue which is hypersensitive to proteolysis, but rather a region of some length. The length of the region of hypersensitivity can be estimated on the basis of the differences in sizes of the large fragments. This region is as large as 11000–13000 M_r. On the basis of the amino acid composition determined by Coleman *et al.* (1977), this represents a sequence of about 100 amino acids, or about 5% of the total protein. These experiments would suggest that this region is part of an exposed surface of the protein and a portion which has no apparent role in any of the three enzyme activities or in allosteric regulation.

In bacteria, the first three enzymes of pyrimidine biosynthesis are on separate proteins which form no complex with one another. In yeast and *Neurospora*, only the carbamoyl-phosphate synthetase and aspartate carbamoyltransferase activities are found covalently linked. The evolution of the CAD-gene locus in higher eukaryotes (including *Drosophila* and mammals) presumably arose from the fusion of at least three separate genes. The fact that a large proteinase-sensitive region lies between the aspartate carbamoyltransferase domain and the rest of the protein might indicate that this region may be a product of the original gene fusion which presumably took place during the evolution of this locus in eukaryotes.

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