*BOVINE CARBOXYPEPTIDASE A VARIANTS RESULTING FROM ALLELOMORPHISM**

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The existence of homologous forms of bovine carboxypeptidase A was suggested by Bargetzi *et al.*[1] on the basis of their observations of a single amino acid replacement in the C-terminal sequence Glu·His·Thr·($\frac{\text{Leu}}{\text{Val}}$)·Asn·Asn. The two species of protein occurred in approximately equal proportions. Since these two variants of carboxypeptidase were isolated from pancreatic tissue pooled from many animals, the question remained whether individual animals (e.g., of different breeds) would produce a single carboxypeptidase. A method developed by Sampath Kumar *et al.*[2] for the isolation of crystalline carboxypeptidase A_α from the pancreas of a single animal revealed both types of proteins in the *one* animal examined.

The present work is an extension of this study to an examination of 15 more animals in an attempt to ascertain whether carboxypeptidase A *ever* occurs as a single protein species in an individual animal. Two explanations could account for the results of Sampath Kumar *et al.* (1) Gene duplication and divergent mutation had occurred, yielding two similar phenotypes (not related as products of two alleles of the same gene, and which might differ considerably in sequence). In this case, both C-terminal peptides would be expected in *all* animals examined. (2) The two enzyme variants represent the products of two alleles of the same gene. The homologous enzymes should then be distributed through the population and segregated in accordance with genetic laws. In this case, the finding of an approximately equal distribution of gene products in the pool would indicate that the alleles would occur with approximately equal frequency within the population, provided that both alleles are fully expressed in the heterozygote. The probability of any one animal being homozygous for one type of carboxypeptidase would be approximately 1 in 4.

This investigation was of particular importance at the present time because of the state of knowledge of the structure-function relationship of this enzyme. Vallee and his collaborators[3] have presented a variety of observations elucidating the nature of the metal ligands, the nature of the substrate-binding site, and the function of critical tyrosine residues in activity. A mechanism of action of the enzyme was formulated on the basis of these data. In addition, current work on the amino sequence of the enzyme in progress in this laboratory[4] by Drs. Babin, Barton, and Srinivasan, and of the three-dimensional structure in crystals by Lipscomb and co-workers[5] should provide the data for a comprehensive evaluation of the relationship between structure and function. Any proved heterogeneity of the material in any of these studies could lead to equivocal conclusions.

Characterization of Carboxypeptidases from Individual Bovine Pancreas Glands.—Fresh bovine pancreatic tissue was obtained immediately after slaughter from 11 animals through the courtesy of Carnation Milk Farm, Carnation, Washington, and Bar-S Meat Products, Seattle, Washington. Crystals of carboxypeptidase A_α were isolated from acetone powders of each individual pancreas by the method of

TABLE 1

COMPOSITIONS OF C-TERMINAL PEPTIDES RELEASED BY CYANOGEN BROMIDE

No.	Breed	Sex	k_0 of Enz. (as HPLA)[a]	Peptide purification procedure[b]	Composition (residues/molecule) Glu	His	Thr	Leu	Val	Asp	Nature of C-terminal peptide
1	Holstein-Friesian[c, d]	Heifer	0.208	2.1	1.01	1.01	0.95	0.43	0.53	1.96	Both types
2	Hereford	Bull	0.188	3:1:1	1.05	0.92	0.96	—	0.96	2.07	Both types
					1.09	0.94	0.97	0.44	0.50	2.03	
				2.1	1.07	0.93	0.99	0.50	0.53	1.97	
3	Brown Swiss	Heifer	0.208	3:1:1	1.25	0.88	1.00	—	0.88	2.00	Both types
					1.14	0.92	0.98	0.71	0.22	2.02	
4	Holstein	Heifer	0.162	3:1:1	1.11	0.27	0.97	0.04	0.92	2.08	Both types
					1.06	0.94	0.97	0.78	0.22	1.97	
5	Hereford	Steer	0.191	3:1:1	1.08	0.96	1.04	—	0.96	2.12	Both types
					1.17	0.96	1.04	0.79	—	2.08	
6	Shorthorn	Steer	—	3:1:1	1.03	0.79	1.03	0.22	0.76	2.10	Both types
					1.17	1.19	0.92	0.83	—	1.90	
7	Holstein-Friesian[d] (3 in number-pooled sample, approx. 3 months old)	Heifer calves	—	3:1:1	1.03	0.94	0.96	—	0.96	2.06	Both types[f]
					1.02	1.02	0.98	0.86	—	2.09	
8	Angus	Heifer	0.198	3:1:1	1.12	0.75	1.02	0.99	—	2.11	Leucine only
				2.1	1.09	0.94	1.02	0.98	—	1.97	
9	Hereford[d]	Bull	0.178	3:1:1	1.02	0.87	1.02	1.02	—	2.05	Leucine only
10	Guernsey	Heifer	0.148	3:1:1	1.10	0.88	1.01	0.96	—	2.02	Leucine only
11	Jersey[d, e]	Heifer	0.208	2.1	1.01	0.96	0.96	1.11	—	1.96	Leucine only
12	Hereford	Heifer	—	3:1:1	1.02	1.06	0.94	—	0.96	2.00	Valine only
13	Brown Swiss	Heifer	—	3:1:1	1.04	1.08	0.96	—	1.00	1.88	Valine only
14	Guernsey[d, e]	Heifer	0.198	2.1	1.10	1.05	0.80	—	0.90	2.15	Valine only

[a] Pure CPAα has a k_0 of 0.208 μmoles min^{-1} μg^{-1}, Bargetzi.[7]

[b] In some cases (2.1) peptides were purified as far as the electrophoresis step (pH 2.1) which does *not* separate the valine and leucine-containing hexapeptides. In other cases (3:1:1) the electrophoresis was followed by chromatography in n-butanol:acetic acid:water. Separation of the two peptides from each other frequently was not quantitative.

[c] These data of Sampath Kumar *et al.*[2] are included for comparison.

[d] Purebred animals.

[e] Obtained from pancreatic juice.

[f] Not included in ratios reported since product of pooled tissue from several calves.

Cox *et al.*[6] as modified by Sampath Kumar *et al.*[2] Pancreatic juice was obtained from two other animals via pancreatic fistulae with the cooperation of Drs. Paul Klaveno and Hugh Butler at Washington State University, Pullman. The juice was fractionated by DEAE-cellulose chromatography, and the partially pure procarboxypeptidase was activated and crystallized according to the method of Cox *et al.*[6] All preparations were recrystallized at least once and the specific activity toward hippuryl-DL-phenyllactic acid (HPLA) was measured by the procedure of Bargetzi *et al.*[7] C-terminal hexapeptides were cleaved from 20 to 40 mg of the purified protein preparations by treatment with cyanogen bromide, following the method of Gross and Witkop,[8] as modified for carboxypeptidase A by Sampath Kumar *et al.*[2] The hexapeptide was purified from the reaction mixture as described previously[1, 2] by selective precipitation of the larger fragments and paper electrophoresis of the small peptide at pH 2.1. In some cases this peptide fraction, located with ninhydrin on a guide strip, was further fractionated by descending paper chromatography in 3:1:1 butanol-acetic acid-water (v/v) for 72 hr. This gave partial separation of the two peptides which were then located by ninhydrin treatment of a guide strip, eluted with water, hydrolyzed for 16 hr in 6 *N* HCl *in vacuo*, and their amino acid compositions determined by the technique of Spackman, Stein, and Moore.[9] The analytical data are summarized in Table 1.

In all cases examined, a peptide fraction was obtained with the expected C-terminal composition. Although both leucine and valine were found in six cases, four cases were found with leucine but no valine, and three cases were found with valine and no leucine in the hexapeptide. These data are entirely consistent with the hypothesis that these two species represent the expression of two alleles of the same gene and that cattle may be either homozygous or heterozygous with respect to this character. Admittedly, the sample of population is too small to determine the statistical frequency of the two alleles (as has been done in the case of β-lactoglobulin[10]); nevertheless, two conclusions may be drawn: first, since one species of enzyme is found in several animals in the complete absence of the other, a segregation of the alleles must be possible and it is highly unlikely that these phenotypes are products of two different genes on one chromosome—as might have arisen by gene duplication; second, there is no obvious relationship between the occurrence of a single species and a biological parameter such as sex, age, or breed of cattle—in contrast to the genetic allelomorphism in the occurrence of the β chain of hemoglobin in cattle[11] wherein only three out of 14 breeds show the variant.

It should be noted that whereas the present mutual replacement of leucine by valine would appear to be a small structural variant and of the "conservative" type as defined by Margoliash and Smith,[13] it is not likely to be a product of code degeneracy since this would be expected in all animals, not just in some. Since no consistent change in specific activity was detected, it is difficult to ascribe enough importance to such a small change in the nature of the amino acid residue to provide selection pressure to increase the gene frequency to 0.5. However, these enzyme activities were only examined with HPLA, a small unnatural (ester) substrate which occupies only a portion of the active site (Vallee[14]). It is therefore important to characterize the specificity of these two molecules further with a variety of substrates in order to explore the effect of amino acid replacement on the whole functional site.

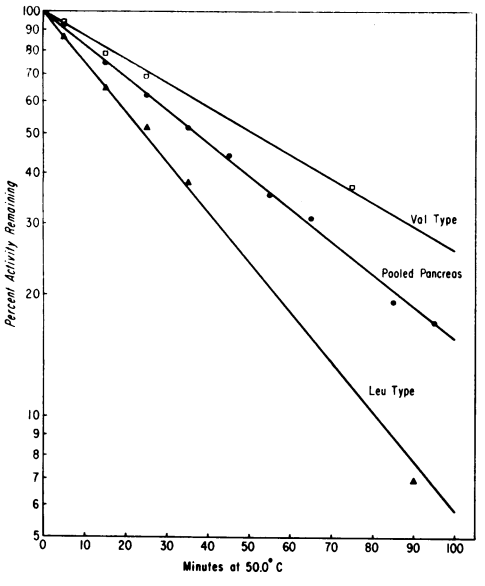

FIG. 1.—Heat inactivation of carboxypeptidase A_α enzymes at 50.0°C in 1 *M* NaCl, 0.005 *M* phosphate, pH 7.50. The enzymes were first dissolved at 0°C in 1 *M* NaCl, 0.5 *M* Tris, pH 8.0 at a concentration of about 20 mg of protein per ml. Pure DFP (5 *M*) was added to a final concentration of 0.05 *M* to preclude traces of endopeptidase activity. After 30 min, the solution was dialyzed against 1 *M* NaCl, 0.001 *M* Tris, pH 8.0 overnight. The enzymes were then crystallized by dialysis against 0.005 *M* phosphate buffer, pH 8.0. The crystals were dissolved in 1 *M* NaCl, 0.005 *M* phosphate, pH 7.50 at a protein concentration of 0.20 mg/ml for use in the heat inactivation study. *Val type* represents valine-type enzyme no. 14, *Leu type* the leucine-type enzyme no. 11, and *pooled pancreas* represents enzyme prepared from pooled pancreata.

Whereas the observed replacement had no significant effect on enzyme activity, the two forms of carboxypeptidase differed measurably in their heat stability in the range 45–60°C. Typical heat inactivation experiments are presented in Figure 1, which shows that the loss in activity follows first-order kinetics. The results suggest that, in analogy with similar studies[15] of the A protein of tryptophan synthetase of *E. coli*, this single amino acid replacement may affect the conformational stability of the entire molecule.

The question remains whether this replacement is the only one within the molecule. Using the method of Ornstein and Davis,[16] heterogeneity can be demonstrated by disk gel electrophoresis. Avreamas and Uriel[17] have recently reported immunoelectrophoretic heterogeneity in the carboxypeptidase of bovine pancreas. The amino acid composition data, given in Table 2, may be compared with those reported previously for the product of a single pancreas[2] and with the pooled material.[7] However, precise as these determinations are, rigid conclusions are on the fringe of reliability of amino acid analyses and small contamination could alter the validity of the interpretation. Nevertheless, the ratios of amino acid residues for the two "leucine enzymes" obtained from two different animals are very nearly identical. In contrast, the ratios for the "valine enzyme" differ significantly from the "leucine enzyme" for some residues. Since the molecular weight cannot be established to the nearest residue, the ratio data are arbitrarily expressed in Table 2 in a way which maximizes the similarities and minimizes the differences. On that basis, differences not exceeding one residue for any one amino acid are found for aspartic and glutamic acids, alanine, isoleucine, and leucine. Of course, another pattern of differences would result from a different basis of comparison.

These small differences between the amino acid ratios of the protein isolated from the homozygous and the heterozygous animals lead us to propose that additional replacement sites may exist within the molecule, thus raising the specter of more than two alleles. We are presently preparing large enough quantities of enzymes from pancreatic juice obtained from fistulae of homozygous animals to explore this

TABLE 2

COMPARISON OF AMINO ACID COMPOSITION* OF SEVERAL TYPES OF CARBOXYPEPTIDASE A_α

	Mean or Extrapolated Residues† per Molecule				
Enzyme Source:	Pooled[7] pancreas	Single[2] pancreas	Single pancreas	Pancreatic juice from one animal	Pancreatic juice from one animal
Animal No. in Table 1:	1	1	8	11	14
Antepenultimate Residue(s):	Val + Leu	Val + Leu	Leu	Leu	Val
Lysine	15.0	14.4	14.6	14.6	14.8
Histidine	8.18	7.6	7.88	7.84	8.13
Arginine	11.1	10.3	10.69	10.61	10.7
Aspartic acid	27.9	27.9	28.5	28.5	28.9
Threonine	27.8	25.1	27.4	25.3	26.0
Serine	33.0	30.7	35.2	31.6	31.9
Glutamic acid	25.0	24.8	25.8	26.2	25.0
Proline	9.70	10.2	9.68	10.0	10.0
Glycine	22.5	22.7	23.1	22.9	23.0
Alanine	(20)	(20)	(20)	(20)	(21)
Valine	15.8	16.0	15.9	16.1	16.1
Isoleucine	20.3	20.0	20.0	20.3	21.1
Leucine	23.2	22.5	24.0	24.2	23.3
Tyrosine	19.2	17.8	18.3	18.3	18.8
Phenylalanine	16.0	15.3	16.1	16.0	16.0

* The data are expressed as amino acid residues per molecule, assuming 20 or 21 residues of alanine per molecular weight of 34,600. The most favorable value for alanine whereby the experimental means of the other amino acids would best approach integral values and where the Val- and Leu-type enzymes would be most similar required two different alanine values.

† Mean of two sets of determinations at four different times of hydrolysis; 24, 48, 72, and 121 hr. Serine and threonine represent values obtained by extrapolation to zero time hydrolysis. Valine and isoleucine represent maximum recovery. Isoleucine values include allo-isoleucine. Cysteine was precluded from the proline peak by air oxidation after hydrolysis. The procedures of hydrolysis and analysis are adopted from Spackman *et al.*[9] and described in detail as applied to carboxypeptidase by Bargetzi *et al.*[7]

possibility and to define the range of carboxypeptidase variants in crystalline preparations from pooled glands.

One further feature of carboxypeptidase to which attention is directed by the present study is the flexibility in structure at both ends of this enzyme which are not accompanied by changes in the specific activity of the molecule. The substitution of leucine for valine in the C-terminal region of the molecule gives rise to no apparent change in activity. This may be compared with the previously observed identical activities of carboxypeptidases A_α, A_β, and A_γ which, in the extreme, differ from each other by as many as seven amino acids at the N-terminus of the molecule.[12]

Summary.—It has been demonstrated that the amino acid replacement in the antepenultimate C-terminal position of bovine carboxypeptidase A_α gives rise to two distinct species of the enzyme which may occur either separately or together in any individual animal. Distribution among 13 animals was shown to be consistent with the approximately equal occurrence of two allelomorphs in the general population. The enzymes were found to have similar activity toward hippuryl phenyllactic acid, but to differ markedly in their heat stability.

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