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ON SUBUNIT II OF BOVINE PROCARBOXYPEPTIDASE A

PROPERTIES AFTER ALKALINE DISSOCIATION

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SUMMARY

Alkali-dissociated Fraction II prepared by an improved technique from bovine procarboxypeptidase A-S6 (procarboxypeptidase A with a sedimentation coefficient of 6 S) was found to be heterogeneous and was resolved into three subfractions; IIa, IIb and IIc by equilibrium chromatography on CM-Sephadex at pH 6.0. Subfractions IIb and IIc could be activated by trypsin to give an N-acetyl-L-tyrosine ethyl ester (ATEE)-splitting endopeptidase whereas IIa was not activated. Subfraction IIb was shown to be an artifact slowly arising from IIc under the conditions prevailing during the dissociation step. The two Subfractions IIb and IIc were observed to have practically the same amino acid composition, sedimentation constant (2.9 S), molecular weight (28 500) and terminal residues (N-terminal half-cystine and C-terminal leucine). All these values demonstrate the very high degree of homology existing between subunit II and porcine chymotrypsinogen C.

Subfractions IIb and IIc were activated at a much slower rate than undissociated subunit II and the k_{cat} of the corresponding ATEE-splitting reaction was also somewhat reduced. However, like activated subunit II, the activated subfractions were found to possess not less than 0.8 active site per mole.

The somewhat lower molecular weight of Subfraction IIa (27 000) and the presence of one N-terminal aspartic acid (or asparagine) pointed out the possibility that this subfraction was a slightly modified form of Fraction III.

INTRODUCTION

Among the three subunits known to exist[1,2] in bovine procarboxypeptidase A-S6 (procarboxypeptidase A with a sedimentation constant of 6 S), subunit I is the

Abbreviations: procarboxypeptidase A-S6 and procarboxypeptidase A-S5, procarboxypeptidase A with a sedimentation constant of 6 S and 5 S, respectively; ATEE, *N*-acetyl-L-tyrosine ethyl ester; DNP-, dinitrophenyl.

zymogen of carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.2.1). Subunit III is still poorly identified. Upon tryptic activation subunit II gives rise to an endopeptidase hydrolyzing the typical chymotrypsin substrate *N*-acetyl-L-tyrosine ethyl ester (ATEE) Like this latter enzyme, the endopeptidase was recently reported to contain an essential serine residue in the sequence Asp–Ser–Gly, an essential histidine[3] and a short chain resulting from the cleavage during activation of the first basic bond in the N-terminal sequence[4]. This chain, however, was found to resemble more that of porcine chymotrypsin C than that existing in bovine and porcine chymotrypsins A and B (EC 3.4.4.5 and EC 3.4.4.6) (ref. 4).

Procarboxypeptidase A-S6 subunits were observed some years ago to be dissociated by a prolonged incubation of the trimer at pH 10.5 (ref. 2) or by succinylation[5]. Succinylated subunits I and II can be activated by trypsin (EC 3.4.4.4) and they are easily separated by gel filtration. But, they have the obvious disadvantage of differing from the native proteins by a varying number of firmly attached succinyl groups. On the other hand, alkaline dissociation may be expected to transform the subunits into more or less degraded forms designated "fractions" by Brown *et al.*[2]. Fraction I, for instance, is irreversibly denatured during the alkaline treatment. The replacement of the N-terminal lysine of native subunit I (ref. 5) by aspartic acid in the fraction[2] is also consistent with the cleavage of one or several peptide bonds. Isolated Fraction II was reported to be activated by trypsin, to give a symmetrical peak by DEAE-cellulose chromatography and to be homogeneous in the ultracentrifuge[2]. But, it was later found to be much less active towards ATEE than activated subunit II in the intact trimer and to contain only about 0.2 active site per mole[3].

It will be shown below that alkali-dissociated preparations of Fraction II include a non activatable compound probably related to Fraction III and two activatable components. Although distinctly less active after tryptic treatment than the undissociated subunit, the latter two were found to contain approximately one active site per mole.

MATERIAL AND METHODS

Purification of procarboxypeptidase A-S6

After some preliminary assays, the procedure finally adopted differed from that of Yamasaki *et al.*[1] by: (a) The addition of 1 mM benzamidine to all solutions and buffers in order to prevent the formation of free trypsin and consequently the early activation of subunit II. The recently identified anionic trypsin[6], which remains with procarboxypeptidase during the first DEAE cellulose chromatography, is especially dangerous in this respect. (b) DEAE-cellulose chromatographies were run at a higher ionic strength and initially at a lower pH in order to individualize better the procarboxypeptidase A-S6 peak. All the experiments were carried out at 4 °C.

Acetone powder (50–200 g) prepared according to Keller *et al.*[7] from freshly collected bovine pancreases was extracted with 10 times its weight of a 1 mM aqueous benzamidine solution and the clear extract was passed through DEAE-cellulose equilibrated with a 50 mM Tris–HCl buffer, pH 7.0. The column was washed by the above buffer containing 60 mM NaCl and procarboxypeptidase A was eluted, as indicated by Fig. 1a, by a linear NaCl gradient of 60 to 250 mM. The top of the peak

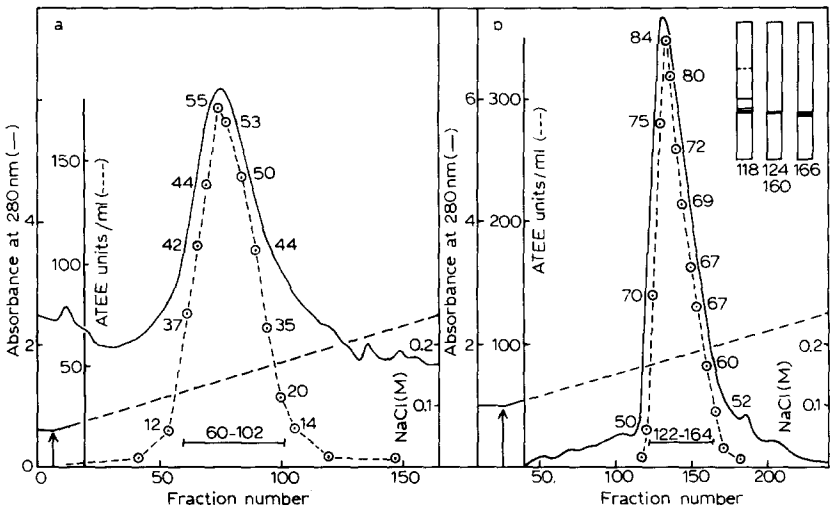

Fig. 1. Purification of procarboxypeptidase A-S6 on DEAE-cellulose. (a) first chromatography of the extract of 50 g acetone powder in a 4 cm × 40 cm DEAE-cellulose column (Brown, fibrous form) equilibrated with a 50 mM Tris–HCl buffer (pH 7.0) in 1 mM benzamidine. After a preliminary washing with the buffer containing 60 mM NaCl, procarboxypeptidase was eluted by a linear gradient of NaCl from 60 to 250 mM. Chamber volume, 2100 ml; fraction volume, 27 ml; flow rate, 120 ml/h. (b) Second chromatography of 4 g of the above lyophilized product obtained in one or several runs. The 5 cm × 40 cm DEAE-cellulose column (Whatman 32, microgranular form) was equilibrated with a 50 mM Tris–HCl buffer (pH 8.0) in 1 mM benzamidine, washed with the same buffer made 100 mM in NaCl and finally eluted by a linear NaCl gradient from 100–250 mM. Chamber volume, 3000 ml; fraction volume, 29 ml; flow rate, 200 ml/h. Disc electrophoresis assays reproduced in the upper right part of b were run at pH 8.6 in a 7.5% gel. Numbers along the peaks designate the potential specific activities of the fractions toward ATEE.

was observed to correspond approximatively to an NaCl concentration of 140 mM. Fractions 60–102 (specific activity with ATEE higher than 20) were pooled and their proteins were concentrated by precipitation in 0.4 saturated ammonium sulfate. The resulting solution was filtered through Sephadex G-25 coarse equilibrated with 5 mM ammonium acetate at pH 6.8, dialyzed against a 50 mM Tris–HCl buffer at pH 8.0 and submitted to a second chromatography on DEAE-cellulose. This chromatography was run at pH 8.0 and the column was washed with 100 mM NaCl before the application of a 100–250 mM gradient of the salt. Fractions 124–160 in Fig. 1b possessed approximately the same potential specific activity towards ATEE and they gave a single band by disc electrophoresis (pH 8.6 and 7.5% gel). These apparently pure fractions were pooled, precipitated as above by 0.4 saturated ammonium sulfate, passed through Sephadex G-25, dialyzed against water and finally lyophilized. The preparations thus obtained were found to be free of nucleic acids and to have a potential specific activity towards ATEE of 70 (average value from 10 determinations). Direct activity was negligible. A high speed ultracentrifugation assay on a solution of 5.4 mg of the lyophilized powder per ml of a 50 mM Tris–HCl buffer (pH 7.0) 100 mM in NaCl resulted in a symmetrical peak from which a sedimentation constant of $s_{20,w} = 5.72$ S could be calculated. The absence of procarboxypeptidase A-S5 (ref. 8) in the preparations was verified by submitting another sample of the solution to a filtration through Sephadex G-200. A single peak was obtained at the

position expected for procarboxypeptidase A-S6. The overall yield (average value from 10 assays) was 1300 mg of pure procarboxypeptidase A-S6 from 100 g of acetone powder.

Preparation of Fraction II by alkaline dissociation of procarboxypeptidase A-S6

The dissociation of procarboxypeptidase A-S6 was carried out as described by Brown *et al.*[2]. A 0.5% solution of the trimer in a pH 10.5 buffer containing 0.28 M LiCl, 0.1 M glycine, 1 mM benzamidine and 10 mM DFP was incubated at 22 °C for 24 h and the pH of the resulting mixture was lowered to 6.0. A precipitate reported to contain denatured Fraction I was removed by centrifugation, the pH was adjusted to 8.0 by dialysis against the 50 mM Tris–HCl buffer and the solution was finally chromatographed on DEAE-cellulose at this pH. Fig. 2 shows that three peaks were

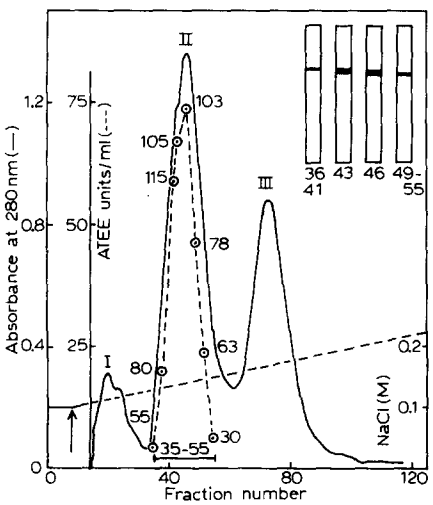

Fig. 2. Isolation of Fraction II. The solution of the alkali-treated procarboxypeptidase A-S6 (500 mg) freed of denatured Fraction I by centrifugation was applied to a 2 cm × 35 cm DEAE-cellulose column (Whatman 32, microgranular form) equilibrated with a 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM benzamidine. The column was washed with the same buffer containing 100 mM NaCl and eluted by a linear NaCl concentration gradient from 100 to 250 mM. Chamber volume, 1000 ml; fraction volume, 13 ml; flow rate, 80 ml/h. The numbers along Peak II indicate the potential specific activity of the fractions towards ATEE. Disc electrophoresis assays were run at pH 8.6 in a 15% gel.

obtained. Peak I could be abscribed to a part of Fraction I remaining in solution. The material under Peak II possessed a strong potential activity towards ATEE and could consequently be identified as Fraction II. The last peak corresponded to Fraction III with perhaps a small amount of undissociated trimer. No peak similar to that designated Peak III' by Brown *et al.*[2] could be discerned on the diagram. The chromatographic fractions 35–55 containing the bulk of Fraction II were pooled, concentrated by precipitation in 0.8 saturated ammonium sulfate, desalted by passage through Sephadex G-25 coarse and lyophilized. The powder thus obtained (about 120 mg) possessed a potential specific activity towards ATEE equal to 100. Direct activity did not exceed 3% of this value.

Zymogen activation, determination of enzymatic activity and radioactivity

In most assays, 0.35–7.0 mg/ml solutions of the zymogens (procarboxypeptidase, Fraction II and Subfraction II) in a 50 mM Tris–HCl buffer (pH 7.8) were activated at 0 °C for 100 min with 1:100 trypsin (weight ratio). To compare the activation course of procarboxypeptidase A and Subfractions II (Fig. 5) the enzyme-substrate ratio was reduced to 1:200.

Activities towards ATEE were measured by titrimetry[9] using 10 mM solutions of the ester in 3% methanol. The molar extinction coefficient of procarboxypeptidase ($\varepsilon_{1\,cm}^{1\%} = 19$ at 280 nm) was employed for calculating the protein concentration of all activated solutions. Potential specific activities were expressed in μequiv. per mg enzyme per min.

Tritiated water and [^{32}P]DFP (specific radioactivity, 0.36 and 16.2 mCi/mmole) were obtained, respectively, from Commissariat à l'Energie Atomique (France) and the Radiochemical Centre Amersham (England). Radioactivity was determined by the scintillation technique in a Packard Tri-Carb liquid scintillation spectrometer Model 3380 after dissolution of the samples in Bray's mixture.

N- and C-terminal residues

N-terminal residues were identified and quantitatively estimated by bidimensional paper chromatography of the ether-soluble dinitrophenyl (DNP) derivatives. The solvents used were *tert*-amylalcohol–2 M ammonia (195:40, v/v) for the first dimension and 1 M sodium citrate (pH 6.2) for the second. The hydrosoluble DNP-cysteic acid was identified by paper electrophoresis (5 V/cm) in 1 M formic acid.

C-terminal residues were determined using 0.5 μmole of reduced-carboxymethylated proteins[10] by the hydrogen–tritium exchange technique in the presence of a basic catalyst[11,12]. At the end of the incubation, the protein was digested by carboxypeptidase and the liberated amino acids were separated in an automatic analyzer Spinco-Beckman Model 120 C. Carboxypeptidase digestion also served sometimes for the direct identification of C-terminal residues and sequences.

Sedimentation constants and molecular weights

Sedimentation constants were determined at 20 °C and 59 800 rev./min with the aid of a Spinco-Beckman ultracentrifuge Model E on 1.5–10 mg/ml solutions of the proteins in a 50 mM Tris–HCl buffer (pH 8.0) 100 mM in NaCl. Molecular weights were measured by equilibrium ultracentrifugation in a short column according to Yphantis[25]. The partial specific volumes were calculated from the amino acid composition of the proteins, except in the case of Subfraction IIc where it was experimentally evaluated[13] with a digital microdensimeter DMA 02C (A. Paar, Graz (Austria)).

Protein molecular weights were determined by three other techniques: gel filtration through Sephadex G-200 column equilibrated with a 50 mM Tris–HCl buffer (pH 7.0) 0.5 M in NaCl[14], filtration of the reduced-carboxymethylated proteins through Bio-gel A-5M in the presence of 6 M guanidinium chloride[15] and zone electrophoresis in 5% polyacrylamide gels in the presence of 1% sodium dodecyl sulfate[16]. The reference proteins used were horse cytochrome *c* (M_r 12 500), bovine trypsinogen (24 000), porcine pepsin (34 000), ovalbumin (43 000) and serum albumin (69 000).

Active site titrations

The activated zymogens in a 50 mM Tris–HCl buffer (pH 7.8) were incubated for 12 h at 0 °C with a 10-fold molar excess of [^{32}P]DFP. Low molecular weight radioactive contaminants were carefully removed by 48 h dialysis against a 50 mM Tris–HCl buffer (pH 7.0) in 500 mM NaCl and by gel filtration through Sephadex G-50 in the same buffer. The number of active sites per mole of protein was calculated by reference to a pure sample of mono diisopropyl [^{32}P]phosphoryl labeled α-chymotrypsin prepared simultaneously with the same labeled DFP. Active site titrations in activated zymogens were also carried out in some instances by evaluating spectrophotometrically at 400 nm the burst of nitrophenolate which occurs when aliquots of the corresponding preparations were mixed at 25 °C with a 0.5 mM *p*-nitrophenyl acetate solution in a 100 mM Tris–HCl buffer (pH 7.5) containing 100 mM NaCl and 50 mM $CaCl_2$. Results obtained by both methods were in fair agreement.

RESULTS

Resolution of Fraction II into three subfractions

Fraction II prepared as described in the preceding Section was distinctly heterogeneous. The central portion of the corresponding peak in Fig. 2 gave 2 bands by disc electrophoresis and the descending limb of the peak had a relatively low potential activity towards ATEE. Fig. 3 shows that this preparation could be resolved into 3 subfractions by an equilibrium chromatography on CM-Sephadex at pH 6.0 The first component emerging from the column (Subfraction IIa) generated after incubation with trypsin a negligible activity towards ATEE. The other two (Subfractions IIb and IIc) were fully activatable. Each component is seen in Fig. 3 to give a single band by disc electrophoresis.

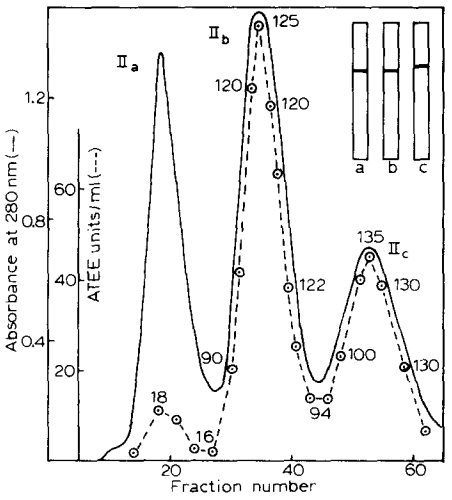

Fig. 3. Equilibrium chromatography of Fraction II on CM-Sephadex G-50. A 170 mg sample of Brown's fraction II isolated on DEAE-cellulose (Fig. 2) was dissolved in 20 ml of a 50 mM Tris–acetate buffer at pH 6.0 and the resulting solution was applied to a 3 cm × 50 cm column equilibrated and eluted by the same buffer. Fraction volume, 9 ml; flow rate, 20 ml/h. Disc electrophoresis were run at pH 8.6 in a 15% gel.

TABLE I

BALANCE SHEET OF THE PROCEDURE LEADING TO THE SUBFRACTIONS

Step	*Protein (mg)*	*Potential units (ATEE)*		*Specific activity (potential)*
		Total number	*(%)*	
Intact procarboxypeptidase A-S6	1000	70 000	100	70
Alkaline dissociation mixture				
Before precipitation at pH 6.0	980	47 000	67	48
After precipitation and removal of Fraction I	690	38 600	57	56
Separation of fractions				
Fraction II	260	26 000	37	100
Fraction III	237	710	1	3
Separation of subfractions				
Subfraction IIa	31	370	0.5	12
Subfraction IIb	83	9 970	14	120
Subfraction IIc	64	8 450	12	132

Results obtained after each step of the preparation leading to the subfractions are summarized in Table I. The two activatable subfractions were observed to contain approximately the same number of potential units (ATEE) and to have about the same specific activity (120–132). The 30% activity loss seen to occur during alkaline dissociation (47 000 units instead of 70 000) will be discussed in more detail later.

Incubation of Subfractions IIb and IIc at pH 10.5

The purpose of this investigation was to find out how the 3 subfractions were related to each other. Lyophilized Subfractions IIb and IIc (10 mg each) were dissolved in 3 ml of the glycine buffer (pH 10.5), 1 mM in benzamidine, used above for the dissociation of procarboxypeptidase A-S6. The two solutions were kept at 20 °C for 24 h, dialyzed against the 50 mM Tris–acetate buffer at pH 6.0 and chromatographed on CM-Sephadex under exactly the same conditions as for the assays depicted in Fig. 3. Fig. 4 shows that Subfraction IIb was not appreciably modified by alkali whereas Subfraction IIc was partly converted into Subfraction IIb. It was noteworthy that neither gave rise to any detectable quantities of Subfraction IIa.

Molecular properties of Subfractions IIb and IIc

Table II indicates that Subfractions IIb and IIc have apparently the same amino acid composition and also the same amide content. Both contain 261–263 residues corresponding to a molecular weight of about 29 000. This composition is also seen to be very similar to that of porcine chymotrypsinogen C (ref. 17). The three proteins contain a single methionine and 5 disulfide bridges. Results are identical for six other residues and very close for seven.

In addition, the three subfractions were observed to be homogeneous by high speed centrifugation. The sedimentation constant ($s_{20,w}$) was found to amount to 2.9 S for Subfractions IIb and IIc and to be significantly lower (2.7 S) for Subfraction IIa. These values were independent from the protein concentration in the 1.5–10 mg/ml range.

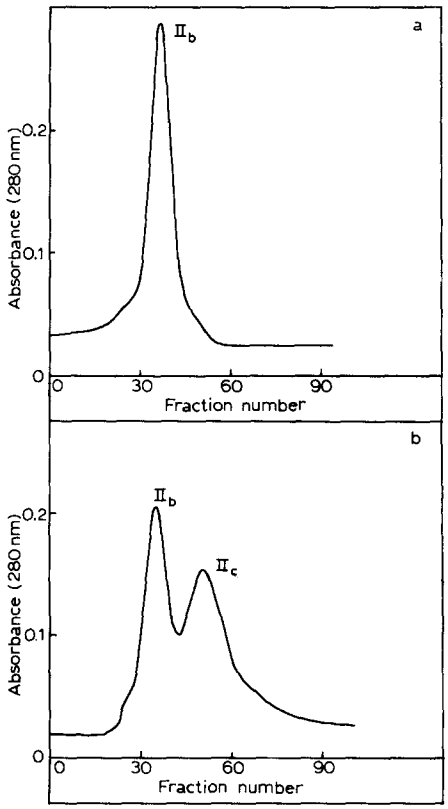

Fig. 4. Alkaline treatment of Subfractions IIb and IIc. (a) and (b), equilibrium chromatography on CM-Sephadex of alkali-treated subfractions IIb and IIc, respectively. For the conditions of the chromatography, see legend to Fig. 3.

The molecular weights of the subfractions were evaluated by several techniques with the results listed in Table III. The best estimate is seen to be 28 500 for Subfractions IIb and IIc and 27 000 for Subfraction IIa. The latter on polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate gave two fainter bands corresponding, respectively, to molecular weight values not exceeding 21 000 and 17 000. As expected, three strong bands were clearly visible when the above technique was applied to trimeric procarboxypeptidase A-S6. The migration rates observed in this case were consistent with the following molecular weights: 40 500 (Subunit I), 28 500 (Subunit II) and 26 500 (Subunit III). If these values are correct, the total weight of the trimer amounts to 95 000.

Finally, half-cystine was found to be N-terminal in Subfractions IIb and IIc by performic oxidation and dinitrophenylation. A C-terminal sequence Leu–Leu was identified in both proteins by a combination of hydrogen–tritium exchange and carboxypeptidase digestion. By contrast, an N-terminal aspartic acid (or asparagine) residue was identified in Subfraction IIa.

TABLE II

AMINO ACID COMPOSITION OF SUBFRACTIONS IIb AND IIc

Residue	*Number of residues per mole of*		
	Subfraction IIb (This work)*	*Subfraction IIc (This work*)*	*Porcine chymotrypsinogen C (Gratecos et al.[17])*
Ala	18	18	15
Arg	9	9	9
Asx	28	28	25
Cys**	10	10	10
Glx	26–27	26	26
Gly	26	26	26–27
His	5	5	6
Ile	14	14	14
Leu	24	24	22
Lys	7	7	7
Met**	1	1	1
Phe	7	7	4
Pro	14	14	13–14
Ser***	16	16	22–23
Thr***	19–20	20	17
Trp†	8	8	12
Tyr	7	7	6
Val	22	21	23
Total number of residues	261–263	261	258–261
Total weight of residues	28 660–28 990	28 760	28 570–28 820
$\varepsilon_{1\,cm}^{1\%}$ at 280 nm	19.4	19.2	23.8
Amides§	23–24	23–24	—

* Next integral number calculated after 24, 48 and 72 h hydrolysis in triple distilled HCl (ref. 18).

** As cysteic acid and methionine sulfone, respectively, after performic acid oxidation.

*** After a linear extrapolation to zero time hydrolysis.

† Colorimetric estimation[19] or determination in the automatic analyzer after an acid hydrolysis in presence of thioglycolic acid[20].

§ By microdiffusion according to Conway[21].

TABLE III

MOLECULAR WEIGHT OF THE SUBFRACTIONS

Technique	*Molecular weight*		
	Subfraction IIa	*Subfraction IIb*	*Subfraction IIc*
Equilibrium ultracentrifugation (Yphantis[25])	—	28 000	27 900
Filtration through:			
Sephadex G-200	27 000	28 000	29 000
Biogel A-5M (in 6 M guanidinium chloride)	27 900	29 900	29 100
Electrophoresis on polyacrylamide gel (in 1% sodium dodecyl sulfate)	26 300	28 000	27 700
Amino acid composition	—	28 800	28 800
Average values	27 000	28 500	28 500

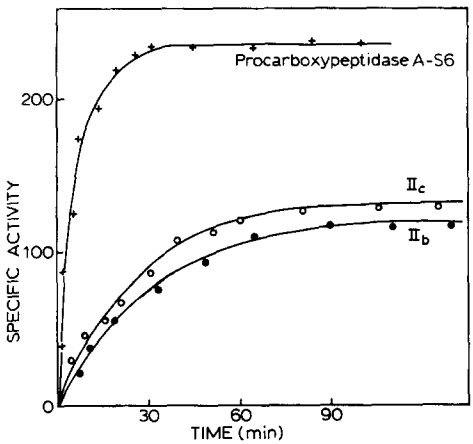

Fig. 5. Activation of subunit II in intact procarboxypeptidase and in the isolated subfractions. The activation conditions are given in text. The true specific activity of subunit II was evaluated approximately by multiplying the value observed for the trimer by the ratio of the molecular weights of the proteins (95 000:28 500).

Activation of the zymogens and active site titrations

As indicated earlier, activation of procarboxypeptidase A-S6, Subfractions IIb and IIc was achieved by incubation at 0 °C and pH 7.8 of 0.35 mg/ml solutions of the zymogens with 1:200 trypsin (weight ratio). The kinetic aspect of the activations is illustrated by Fig. 5. Fig. 5 shows in the first place that intact procarboxypeptidase attains a higher maximal specific activity than the isolated subfractions and that it is activated much faster.

It was also interesting to find that activated procarboxypeptidase and subfractions incorporated upon [^{32}P]DFP treatment the same amount of radioactivity per mole and that this amount was just a little lower (80%) than the one measured in a purified sample of mono diisopropyl [^{32}P]phosphoryl-labeled α-chymotrypsin prepared with the same labeled DFP. Moreover, when mixed with *p*-nitrophenylacetate as previously described, the three activated zymogens induced a burst of *p*-nitrophenol equal to 0.85–1.02 moles/mole. The burst observed with activated bovine chymotrypsinogen A was 1.07 moles/mole and it rose to 1.23 moles/mole after a purification of the resulting chymotrypsin by affinity chromatography[22] Therefore, it could be concluded unambiguously that activated Subfractions IIb and IIc as well as activated procarboxypeptidase possessed approximatively one active site per mole. These latter enzymes could not be purified by affinity chromatography because of their insolubility at acidic pH.

DISCUSSION

Fraction II, prepared according to Brown *et al.*[2] from alkali-dissociated bovine PCP A-S6, was found in this work to be composed of three subfractions; IIa, IIb and IIc. Subfractions IIb and IIc could be activated by trypsin and transformed into an ATEE-splitting endopeptidase. Subfraction IIa was not activated under the same conditions.

Subfractions IIb and IIc, which readily separated on CM-Sephadex under equilibrium conditions must be considered as distinct entities. However, their molecular parameters including amino acid composition, amide content, sedimentation constant, molecular weight, N- and C-terminal residues were found to be practically identical. They turned out to have also the same potential activity towards ATEE. Although the number of amide groups was found by analysis to be exactly the same in both proteins, it is not excluded that this number actually differed by 1 or 2 units. Amide groups are known to be very labile at alkaline pH values. Another, less likely, hypothesis was that Subfraction IIb arose from Subfraction IIc by an alkali-induced conformational transition "exposing" or "burying" a few charged groups in the molecules.

It was also interesting to note that, in contrast to bovine carboxypeptidases A, Fractions IIb and IIc were not produced by allelomorphic genes[23]. Both forms were found in similar proportions in 8 distinct preparations starting each from a single pancreas. Moreover, the diagrams presented in Fig. 4 demonstrate that Subfraction IIb was an artifact generated by Subfraction IIc during the dissociation.

By contrast, Subfraction IIa was found not to arise from either Fraction IIb or IIc. Its lower molecular weight and the presence in this compound of an N-terminal aspartic acid (or asparagine) led to the assumption that it was derived from Fraction III by a still unknown mechanism.

The additional purification effect exerted by the removal of Subfraction IIa from those directly related to subunit II reinforced the early hypothesis[4,24] of a high degree of structural homology between this subunit and porcine chymotrypsinogen C (ref. 4). The amino acid composition of the proteins were shown to be identical for 8 residues and very close for 7. Table II also indicated that porcine chymotrypsinogen C contained more serine than the bovine subfractions but less threonine, more tryptophan but less phenylalanine. Moreover, the proteins had practically the same number of residues and the same molecular weight (about 28 500) which was distinctly higher than that of chymotrypsinogens A and B (about 25 000). All these facts strongly suggest that the proteins have an identical sequence except for a limited number of mostly conservative substitutions. Molecular weight values of 25 000 and 27 000 had been proposed, respectively, for succinyl Fraction II after purification on hydroxylapatite[3] and for alkalidissociated Fraction II (ref. 2).

Another point of interest is related to the activability of isolated fractions or subfractions when compared to that of native subunit II. Alkali-dissociated Fraction II was recently found by Behnke *et al.*[3] to possess a substantially lower potential activity towards ATEE than native subunit II. This finding was correlated with a 6-fold drop of the reaction maximal velocity. The number of active sites per mole that could be titrated by several techniques in α-chymotrypsin, undissociated subunit II and Fraction II were, respectively, 0.8, 0.6 and < 0.2. It was confirmed in this work that the potential activity of Subfractions IIb and IIc was lower than that expected for native subunit II. However, the k_{cat} (130 s^{-1}) was not found to differ by more than 40% from that calculated for the reaction catalyzed by the subunit. In addition, not less than 0.8 active site per mole was titrated by two techniques in the isolated subfractions as well as in subunit II. This higher and more consistent result is due in part to the removal of the inactive Subfraction IIa and probably also to a better protection of Subfractions IIb and IIc during the dissocia-

tion step and further purification. It makes it unnecessary to postulate[3] the presence of more than one subunit II in procarboxypeptidase A-S6. The really significant difference between subunit and Subfractions II was encountered here at the level of the activation rate which, as indicated by Fig. 5, was much lower for the subfractions. The origin of this interesting difference is now being studied.

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