BIOGENIC AMINE SPECIFICITY OF CORTICAL PEPTIDE SYNTHESIS IN MONKEY BRAIN

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1. Introduction

N-acetyl aspartic (Acetyl-Asp) is present in mammalian brain tissue at a concentration of 5–6 μmoles/g fresh tissue weight [1,2]. The compound was previously considered to be metabolically inert [1], and has a very slow turnover in cortex slices in vitro [3] and in vivo [4]. A rapid sequestration of Acetyl-Asp into an alkali-labile compound on addition of mono-, di-, or poly-amines to tissue slices of brain [5], and stoichiometric with the added amine, suggested that Acetyl-Asp could take part in a cyclic process without being metabolized. Blocking monoamine oxidase (EC 1.4.3.4) in homogenates of mouse brain incubated in the presence of amino acids (1 mM), an ATP-regenerating system and histamine, led to the formation of numerous *N*-acetyl-aspartyl peptides [6]. These were formed de novo as judged by isotope incorporation from ^{14}C-labelled and recoverable precursors, and probably independently of protein synthesis as the formation was not affected by inhibitors of the protein synthesizing machinery of the tissue [6]. We wish to report that a certain degree of specificity of peptide synthesis, dependent on the amine added, is discernable in homogenates of monkey cortex, and that the pattern of peptides formed is regulated to a large extent by the amino acids present in concentrations above 0.5 mM in the homogenate.

2. Materials and methods

Brains of *Cercopithecus ethiops* were obtained and stored at −90°C as described previously [7]. The frozen cortical tissue was forcibly cut into thin flakes and homogenized for 20 sec at 715 rpm in a Potter Elvehjem Teflon homogenizer with all-round clearance of 0.01 cm in 4 vol of ice-cold phosphate buffer [8] containing 4 mM Nialamide.

Incubation was performed for 30 min as described [6] with an ATP-regenerating system. Final concentrations in the incubation medium were K-phosphate 25 mM, pH 7.4, $MgCl_2$ 10 mM, Glucose 10 mM, Na-phosphate 5 mM, pH 7.4, KCl 50 mM, ATP 0.5 mM, phosphoenolpyruvate 0.25 mM and pyruvate kinase (EC 2.7.1.40 from rabbit muscle) 20 μg/ml. The concentration of each amino acid added was 0.5 mM and of monoamines 0.5 to 1 mM. The reaction was stopped with perchloric acid to 0.1 M at 0°C and the perchloric acid removed as K-perchlorate by neutralisation with KOH to pH 6.5.

The amino acids and peptides with free N-terminal amine groups were removed by cation exchange on Dowex 50 × 2 (200–400 mesh, 1 × 10 cm) in the H^+ form. The *N*-substituted amino acids and peptides were separated by anion exchange as described in fig. 1 and ref. [6]. Individual peaks were lyophilized and passed through a Bio-Gel P 2 column (BIORAD Labs: 100–200 mesh with exclusion limit 1600 daltons) of dimensions 1.6 × 90 cm in 0.2 M formic acid at a flow rate of 2 ml/10 min. Further purification was carried out by chromatography on washed Whatman 3 MM paper [7] and silica gel plates in *n*-butanol/acetic acid/water (120:30:50; by vol) and phenol/water (250:62,5; weight: volume). Complete hydrolysis was performed in 6 M glass-distilled HCl at 110°C for 16 hr [6], the HCl removed under vacuum over KOH, and the residue analyzed on a commercial amino acid analyzer [6]. Partial fractionation and cleavage of especially the labile aspartylpeptide bond was performed in 0.1 M HCl for 1 hr at 110°C, followed by anion

exchange as described in fig. 1, thin-layer chromatography and complete hydrolysis of the fragments. When a constant amino acid composition was obtained, despite further purification with concommitant loss of material, the peptides were considered tentatively pure. ^{14}C-labelled Acetyl-Asp (specific activity 71 200 cpm/μmol) was prepared as described [6] and incorporation measured in Asp and or Acetyl-Asp recovered from the peptides after hydrolysis [6].

3. Results

Fig. 1 illustrates the pattern of *N*-substituted compounds obtained with either Noradrenaline or Dopamine in the presence of 0.5 mM Acetyl-Asp, Glu, GABA and Cys added to cortical homogenates. In the absence of an ATP-regenerating system or monoamine no peptides were formed in monkey and mouse cortex [6]. Peak C has previously been shown to be pyroglutamic acid [6,7]. The peak appearing at 220–250 ml (fig. 1) is a complex peak consisting of cysteine and taurine-containing peptides. Peak A and B moved as single peaks on gel filtration on P2 with the major component of each eluting earlier than free amino acids and amines. Peak A had an apparent K_{av} of 0.49, B of 0.51, while Acetyl-Asp had a K_{av} of 0.78. On thin-layer chromatography both A and B moved as one spot as determined by the labelling from the Acetyl-Asp which was then recovered by hydrolysis in 0.1 M HCl for 1 hr, together with Asp and other peptide fragments (table 1). The total amino acid

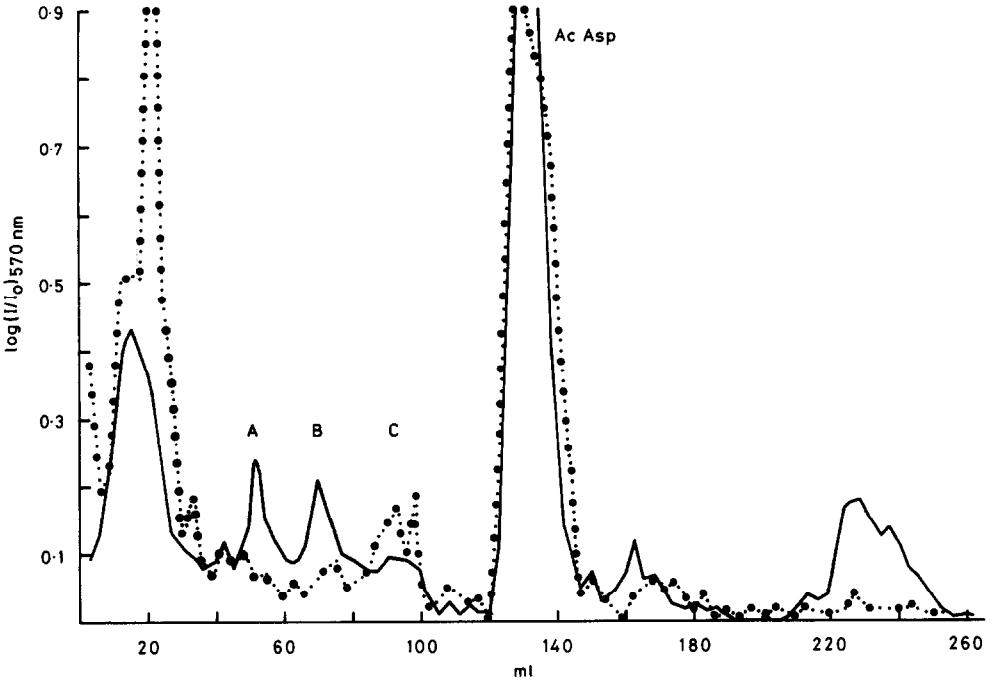

Fig. 1. The amino acids and peptides with a free amino terminal group except Tau, were removed by cation exchange at pH 6.2 as described in methods. After freeze drying, dissolving in 3 ml H_2O and neutralizing to pH 6.5, the *N*-substituted compounds were applied on to a Dowex 1 X 4 (200–400 mesh) column in the formate form of dimensions 1 X 20 cm, and eluted with a formic acid gradient of water to 2 M formic acid (200:200 ml by vol) at a rate of 2 ml fractions/10 min [7]. The *N*-substituted compounds were split by hydrolysis of 0.4 ml aliquots of each 2 ml fraction in 2 M KOH for 2 hr [6], neutralized by HCl and reacted with ninhydrin using Rosen's technique [9]. Without hydrolysis only Tau, eluting at 10–20 ml was coloured. A log I/I_0 at 570 nm of 0.210 is equivalent to 0.05 μmoles of amino acid. ——— is the curve obtained with 0.5 mM Noradrenalin, and –•–•–•–• the curve for 0.5 mM Dopamine with Acetyl-Asp, GABA, Glu and Cys (1 mM) added to the incubation mixture. Log $(I/I_0)_{570\ nm}$ range in 3 experiments for peak A was 0.250–0.400.

Table 1

From fig.	Elution vol (ml)	Isolation sequence	K_{av}	R_f	Composition	n	0.1 M HCl fragments	Probable structure	mmol/mol Ac-Asp in 30 min
1	A (50–60)	1	0.49	0.13	Asp^4, Gly, Cys	3	Ac-Asp	Ac-Asp (Asx^3, Cys, Gly)	n.d.
1	B (67–75)*	1	0.51	0.31	Asp, Glu Ser, Gly	3	Ac-Asp, Ac-Asp–Glu, Glu–Ser	Ac-Asp–Glu–Ser–Gly	n.d.
2	43–55	1	0.32	0.76	Asp, Ser^2, Glu^2, Gly^2, Ala	4	Ac-Asp, Ac-Asp–Glu	Ac-Asp–Glu (Ser^2, Glu, Gly^2, Ala)	n.d.
2	155–164*	1 and 2	0.32	0.15	Asp, Glu, Gly^4, Cys^2, Tau	5	Ac-Asp, Ac-Asp–Glu	Ac-Asp–Glu (Gly^4, Cys^2) Tau	54-110 $n' = 3$
2	155–164*	1 and 2	0.55	0.65	Asp, Glu	10	Ac-Asp, Asp–Glu	Ac-Asp–Glu	67-99 $n' = 4$
2	160–180*	1 and 2	0.50	0.54	Asp^2, Glu	5	Ac-Asp, Ac-Asp–Glu	Ac-Asp–Glu–Asp	n.d.
2	160–180*	1 and 2	0.35	0.59	Asp^4, Ser, Gly, Glu	4	Ac-Asp–Asp	Ac-Asp–Asp (Asx^2, Glu, Gly, Ser)	n.d.

Fractions were pooled and corresponding fractions from 5–10 experiments added together. Purification sequence 1 involves the following: Dowex 50 – Dowex 1 – P 2 – paper – TLC × 2; and sequence 2: Dowex 50 – Dowex 1 – paper – P 2 – TLC × 2 (see Matherials and methods). Rf values are given for paper chromatography in n-Butanol/acetic acid/water. $K_{av} = V_e - V_0 \times 1/V_t - V_0$. n is number of amino acid analyses after hydrolysis. The more important fragments from 0.1 M HCl, 1 hr, 110° C hydrolysis and anion exchange on Dowex 1 (as in fig. 1) are shown. Incorporation is calculated from specific activity of ^{14}C-labelled Acetyl-Asp (Ac-Asp in table) found in Asp or Acetyl-Asp following 0.1 M hydrolysis [6]. Asp, Acetyl-Asp and Glu have $K_{av} = 0.78$.
* The compound has been reported in mouse brain [6]. n.d. not determined.

composition of the purified peptides is shown in table 1, where the range of incorporation of Acetyl-Asp is also given.

Fig. 2 demonstrates the pattern obtained with histamine (0.5 mM) and the amino acids added as in fig. 1. If however, histamine is incubated in the cortical homogenates with Acetyl-Asp, Glu, Gly, Ala, Tau, $GluNH_2$, Cys and Ser added (0.5 mM), a complex elution pattern with numerous peptidic peaks is demonstrable (fig. 2). The compositions of a few of these peptides are shown in table 1. K_{av} values for gel filtration on P2 gel are given in a separate column, and are clearly closer to the void volume than free amino acids. It should be noted that several of the peptidic constituents are transmitter candidates, or have effects on membrane potentials, such as Asp, Glu, Gly, Tau, Cys and Ala.

4. Discussion

The compounds are peptides as they are separable from the constituent free amino acids by ion-exchange, gel filtration, paper and thin-layer chromatography. These peptidic compounds are furthermore N-substituted as all free amino acids except Tau are retained on a Dowex 50 column in the H^+ form at pH 6.5, and also Acetyl-Asp could be recovered from the compounds on partial hydrolysis. Furthermore the compounds were were ninhydrin positive only after hydrolysis (fig. 1). The bonding must be covalent in order to survive the sequence of isolation which exposes the compounds to low pH, and, during elution from the thin-layer chromatograms, to low ionic strength [10]. The compounds are also probably formed de novo as they incorporate ^{14}C-labelled Acetyl-Asp and also other ^{14}C-labelled amino acids (to be published). The simple molar ratio

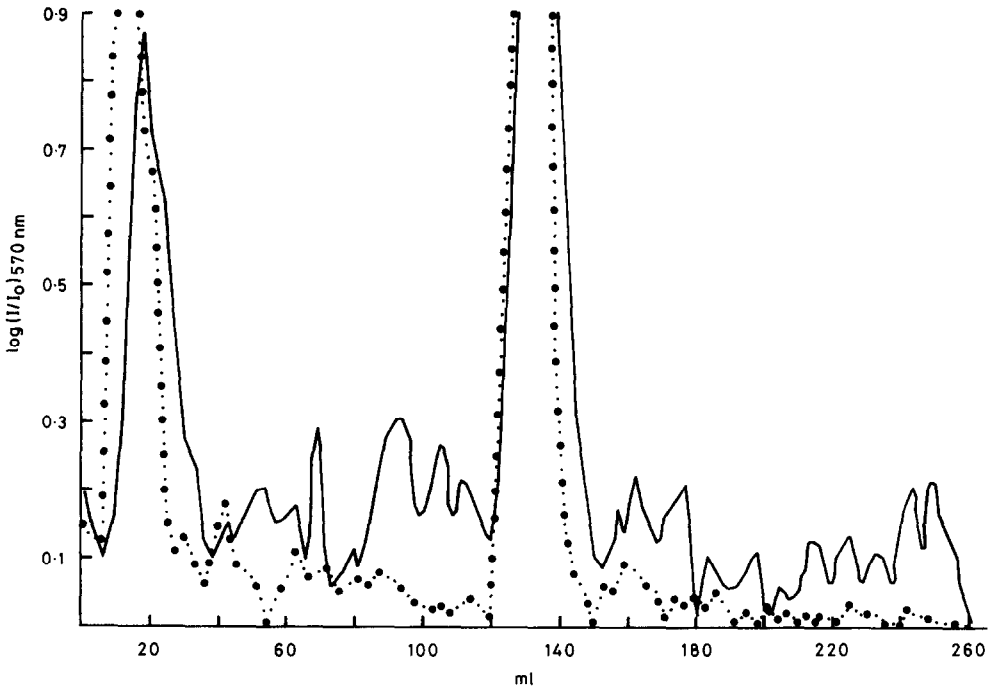

Fig. 2. Anion exchange on Dowex 1 of the *N*-substituted peptides and amino acids, formed with the amine histamine (0.5 mM) present. For (●–●–●–●) the amino acids Acetyl-Asp, Glu, GABA and Cys (1.0 mM) were used. (———) Demonstrates the vastly more complex pattern obtained when Acetyl-Asp, Glu, Gly, Ala, Tau, Gln, Cys and Ser (1 mM) were added to the incubation mixture. Without prior hydrolysis in alkali, no ninhydrin colour was formed except at 10–20 ml (Tau).

of the constituent amino acids, which remains constant in spite of further purification with loss of material, suggests that the peptides probably are pure. One of the peptides, Acetyl–Asp–Glu is found preformed in brain [4], and the isolated peptide behaved as synthetic standards.

The function of the peptides, formed in cortical homogenates is unknown. It may be relevant that the hypothalamic releasing factors, contained in vesicles (granula) of the median eminence [11] are also peptides [12], and that the hypothalamus contains high endogenous levels of monoamines [13]. Monoamines are also associated with polypeptide hormones and propeptides in the storage granula in endocrine cells [14]. The peptide synthesis reported here, previously shown to be probably independent of protein synthesis [6], is apparently largely determined by the amine present, as well as the amino acids added at concentrations above 0.5 mM in the medium. Data from a more purified enzyme complex supports this view (to be published).

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