ENKEPHALIN ANALOGS. INTRODUCTION OF STEREOCHEMICAL CONSTRAINTS, METAL BINDING SITES AND FLUORESCENT GROUPS

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1. Introduction

There has been considerable speculation on the biologically active conformations of the enkephalins and their possible structural similarity to the opiates [1–4]. A number of models have been proposed on the basis of theoretical calculations [2] and computer modelling [3]. Recent NMR studies suggest a high degree of conformational flexibility at Gly^2 and Gly^3 implying that a favoured conformation does not exist in aqueous solution [5]. The replacement of the Gly residues by Aib residues will greatly restrict the available conformations at positions 2 and 3 of the pentapeptides and may thereby allow a better definition of the steric requirements for biological activity. This approach appears particularly attractive in view of the extremely well defined conformations adopted by Aib containing peptides [6–10]. An earlier report described the synthesis of Aib^2–Met^5–enkephalinamide [11]. Here we present the preparation and compare the biological properties of the Aib^2, Aib^3 and Aib^2–Aib^3 Met^5–enkephalin derivatives. The properties of 3-nitro–Tyr analogs, designed to bind paramagnetic NMR shift probes and a fluorescent Aib^2 analog developed for studies of receptor interactions, are also described.

2. Experimental procedures

Peptides listed in table 1 were synthesised by solution phase methods using DCC or DCC/1-hydroxybenzotriazole-mediated couplings. Boc groups and methyl esters were used for amino and carboxyl protection, respectively. Removal was effected with HCl–tetrahydrofuran or trifluoroacetic acid for Boc groups and saponification in 2 N NaOH–CH_3OH for the esters. Analogs with Aib at residue 2 (2,3,6,7) were prepared by **2+3** condensation, while Gly^2 analogs (1,4,5) were obtained by **1+4** coupling. The DNS group was introduced by coupling Boc–Met to DNS–NH–CH_2–CH_2–NH_2 [12] followed by Boc removal and condensation with Boc–Gly–Phe to yield the fluorescent tripeptide fragment. The 3-nitro–Tyr and DNS analogs (5,6,7) were purified by preparative TLC on silica gel using **8:2** $CHCl_3$–CH_3OH for 5 and 6 and 9:1 $CHCl_3$–CH_3OH for 7. All analogs were checked for homogeneity by TLC on silica gel using 8:2 $CHCl_3$–CH_3OH or **4:** 1:1 n-butanol–water–acetic acid systems. Faint iodine positive but ninhydrin-negative spots with higher R_F were present in 2 and 3. 1,2,5,6 and 7 gave 270 MHz ^1H NMR spectra fully consistent with their structures, while **3** and **4** yielded satisfactory 60 MHz spectra.

The biological activity of enkephalin analogs was tested by microinjection of the peptides into the lateral ventricle of the brain of adult albino mice in groups of 4. Mice were anaesthetised by brief exposure to ether prior to injection. The control mice received an equal volume of the diluents, alcohol and water without the peptide. Alcohol was necessary to solubilise certain analogs at neutral pH. Injection of enkephalins immediately immobilised the mice and also had a pronounced sedative effect. The mice assumed abnormal postures during the period of sedation, salivated profusely and in a few teeth chattering was evident. Subsequently the mice overcame

Abbreviations: Aib, *a-aminoisobutyryl; Boc, t*-butyloxy*carbonyl;* **DCC, dicyclohexylcarbodiimide; DNS,** 1-dimethyl**aminonaphthalene-5-sulfonyl; TLC,** *thin-layer chromatography*

Table 1
Effect of **enkephalin analogs on mice**

Samples injected[a]	Dose (μg)	Mean recovery time (min)[b]
Control (1:7 alcohol–water)	–	2
Control (1:2 alcohol–water)	–	4
Tyr–Gly–Gly–Phe–Leu–NH, 1	100	43
Tyr–Aib–Gly–Phe–Met–NH, 2	50	140
Tyr–Aib–Aib–Phe–Met–NH, 3	50	140
Tyr–Gly–Aib–Phe–Met–NH, 4	100	21
(3-NO_2)Tyr–Gly–Gly–Phe–Met–NH, 5	100	25
(3-NO_2)Tyr–Aib–Gly–Phe–Met–NH_2 6	100	63
Tyr-Aib-Gly-Phe-Met-NH-fCH,), -DNS 7	50	100

[a] Injected volumes were 6 μl except for 2 where it was 3 μl. All samples were injected in 1:7 alcohol–water solution, except 7 which was dissolved in 1:2 alcohol–water

[b] Recovery times are averaged for groups of 4 mice

this effect but appeared drowsy and moved very sluggishly or in jerks with a completely inactive or limping hind leg. A time lag persisted before the mice attained normal Coordinated limb movement. In the present investigation the biological activity of the various analogs was monitored by comparing the recovery time of the mice from the effect of enkephalins. Recovery time is the interval that lapses from the point of administration of the enkephalins to the complete recovery of the mice, as judged by the attainment of normal coordinated movement of the limbs, as seen in normal mice.

3. Results and discussion

Table 1 summarises the biological activity of the peptides, as measured by the recovery of coordinated movement in mice. It has earlier been demonstrated that direct injection of β-endorphin into the brain of rats results in marked behavioral effects. Enhanced salivation [13], pronounced sedation and a state of immobility without motor paralysis [13–15] have been observed. The state of catatonia was reversed by the opiate antagonist, naloxone [14]. We have therefore chosen to follow the mean recovery time for normal movement, after direct injection into the brain of mice, as a parameter for comparing the activity of the synthetic peptides. The results in table 1 clearly demonstrate that the Aib^2 and Aib^2–Aib^3 3 analogs are significantly more active than Leu^5–enkephalinamide 1. The replacement of Met' for Leu^5 has only a small effect on activity. While all peptides were tested upto a concentration of 100 μg, the injection of 3 at these levels led to death of the animal. Consequently the more active analogs were also examined at the lower dose of 50 μg.

The pronounced enhancement in the activity of the enkephalins on replacement of Gly^2 and Gly^3 by Aib residues suggests that the active conformations of the natural peptides are indeed accessible to the more hindered synthetic analogs. Earlier studies have established the tendency of Aib residues to initiate β-turns [6–10]. X-Ray diffraction [6–8], infrared [9] and NMR [10] studies have shown that –Aib–Aib– and –Aib–X– sequences favour β-turn structures, stabilised by intramolecular 4 ← 1 hydrogen bonds, in non-aqueous solution. Theoretical studies suggest that ϕ, ψ values [16] close to the 3_{10} and a-helical regions of the conformational map are preferred for Aib residues [17,18]. An examination of crystal structures of Aib containing peptides [6–8,19,20] yielded a total of 11 Aib residues all of which had ϕ, ψ values confined to the regions ϕ $-60 \pm 20°$, ψ $-30 \pm 20°$ or ϕ $+60 \pm 20°$, ψ $30 \pm 20°$. It is therefore likely that the Aib analogs adopt a well defined backbone conformation. Preliminary evidence for a 4 ← 1 hydrogen bond

in 2 involving the C=O group of Tyr' and the N–H group of Phe[4] has been obtained from 'H NMR. The temperature coefficient of the Phe NH chemical shift in $(CD_3)_2SO$ is found to be 2.77×10^{-3} ppm/°C whereas the corresponding values for the Gly–NH and Met–NH groups are 5.55×10^{-3} and 4.63×10^{-3} ppm/°C, respectively. The value obtained for Phe NH in Met[5]–enkephalinamide is 4.63×10^{-3} ppm/°C (T. S. Sudha, unpublished results). Interestingly, such a hydrogen bond is indeed observed in the crystal structure of Leu[5]–enkephalin, with conformational angles of $\phi = 59°, \psi = 25°, \# = 97''$ and $\psi = -7°$, for residues 2 and 3 [21]. These values are generally stereochemically favourable **for** D-amino acids and the optically inactive Aib residues can indeed adopt these values. The β-turn has also been proposed in earlier studies [1].

The conformations possible for the Aib[2]–Aib[3] analog 3 are further restricted relative to 2. The dramatic enhancement in biological activity of 3 compared to 1 suggests that the active conformations may require values of ϕ_2, ψ_2 and ϕ_3, ψ_3 in the range $\phi = +60 \pm 30°$ and $\psi = +30 \pm 30''$. The choice of the positive ϕ, ψ values **is** made in view of the very low receptor affinity of L-Ala[2]–enkephalinamide compared to the D-Ala[2] or Gly[2] peptides [22]. Studies fitting the enkephalin structure to a proposed opiate pharmacophore suggested a biologically active conformation having $\phi_2 = 160°, \psi_2 = -87°, \phi_3 = -118''$ and $\psi_3 = 98°$ [3]. These values represent significantly higher energy conformations for both 2 and 3. It is unlikely that favourable receptor interactions will offset this destabilisation. Modelling studies have also predicted that the Aib[3] analog 4 will be inactive [23]. The results in table 1 show that 4 possesses substantial activity, though in this test system the activity is lower than Leu[5]–enkephalinamide. From conformational considerations the high activity of the derivatives 1, 2, and 3 implies that **4** should also exhibit activity. In view of the known lability of the Tyr[1]–Gly[2] bond to enzymatic degradation [22] a direct comparison of the analogs with Aib[2] and Gly[2] may not be strictly valid, under the conditions of testing.

The binding of metal atoms at specific sites in biomolecules permits application of the NMR shift probe method to the study of solution conformations in aqueous solution [24]. It has been proposed that 3-nitro–Tyr can serve as a metal binding site in proteins [25] and an application of the method to the study of bovine pancreatic trypsin inhibitor has been reported [26]. In order to explore the potential of this method we have synthesised the nitrotyrosine analogs 5 and **6**. It is seen from table 1 that introduction of nitro group *ortho* to the phenolic hydroxyl, reduces activity by a factor of two compared to 1 and 2. The retention of substantial activity suggests that the nitro group does not completely impede the receptor interaction of the phenolic sidechain. The ***ortho*** nitro group would also reduce the pK_a of the phenol from a value of 10.1 in Tyr to a value of 7.2 in nitrotyrosine [27]. **As** a consequence ionisation of the phenolic function is possible at physiological pH. Whether such a process is responsible for the observed attenuation in activity remains to be established. Preliminary NMR studies suggest a gross overall conformational similarity between 2 and 6. The nitrotyrosine derivative may therefore be used to further develop details of the three-dimensional structure of the Aib[2] analogs in solution.

Fluorescent enkephalin derivatives may prove useful in probing opiate receptors and enkephalin binding sites in subcellular fractions from brain tissue. Table 1 shows that introduction of a dansyl group, coupled via an ethylene-diamine spacer to the Met[5] carboxyl group leads to an active, fluorescent derivative 7. This derivative may also be used to probe molecular conformation by monitoring energy transfer [4] from Tyr[1] to the dansyl moiety. During the course of our studies a report on the synthesis of active fluorescent analogs of the Gly[2]–Gly[3] and D-Ala[2]–Gly[3] Met[5]–enkephalins appeared [28]. A comparison of energy transfer efficiencies in the Gly[2] and Aib[2] derivatives may yield further information on peptide folding. Detailed conformational analysis of the peptides reported here together with a more comprehensive study of their biological activity may be of value in establishing structure–function correlations for the enkephalins.

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