

Stabilization of β -turn conformations in enkephalins

α -Aminoisobutyric acid analogs

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Stereochemical constraints have been introduced into the enkephalin backbone by substituting α -aminoisobutyryl (Aib) residues at positions 2 and 3, instead of Gly. ^1H n.m.r. studies of Tyr-Aib-Gly-Phe-Met-NH₂, Tyr-Aib-Aib-Phe-Met-NH₂ and Tyr-Gly-Aib-Phe-Met-NH₂ demonstrate the occurrence of folded, intramolecularly hydrogen bonded structures in organic solvents. Similar conformations are also favoured in the corresponding *t*-butyloxycarbonyl protected tetrapeptides, which lack the Tyr residue. A β -turn centred at positions 2 and 3 is proposed for the Aib²-Gly³ analog. In the Gly²-Aib³ analog, the β -turn has Aib³-Phe⁴ as the corner residues. The Aib²-Aib³ analog adopts a consecutive β -turn or 3_{10} helical conformation. High *in vivo* biological activity is observed for the Aib² and Aib²-Aib³ analogs, while the Aib³ peptide is significantly less active.

Key words: α -aminoisobutyryl peptides; enkephalin analogs; conformational constraints; peptide conformation; β -turns.

Enkephalins (Tyr-Gly-Gly-Phe-Met/Leu) are endogenous brain peptides with morphine like activity (1). Considerable effort has been directed towards establishing the structural and conformational requirements for pharmacological activity. Numerous studies have been undertaken, both experimental (2-9) and theoretical (10-12), to elucidate the nature of the receptor bound conformations. While there is a consensus that enkephalins are compact and folded at the receptor site, considerable disagreement exists on the specific nature of the proposed structure. Theoretical studies have provided some support for Gly²-Gly³ β -turn structures (10)(Fig. 1a). A Type I' Gly²-Gly³ β -turn has been observed for Leu enkephalin in the solid state (9). Spectroscopic

studies, in solution, have also been interpreted in terms of a Gly³-Phe⁴ β -turn (2-7)(Fig. 1b).

Some investigators have also suggested that enkephalin structures are highly flexible, in solution, with no particular conformational preference (8). The Gly-Gly segment contributes considerably to this backbone flexibility. Studies of active stereochemically constrained enkephalin analogs may be of value in developing structure-activity correlations. In the present work, α -aminoisobutyryl (Aib) residues are introduced instead of Gly² and Gly³, individually and simultaneously, in an attempt to narrow down the range of backbone conformations recognized by opiate receptors. The choice of Aib residues was dictated by their ability to restrict peptide backbone freedom

(13–15), with the concomitant stabilization of β -turn structures (13, 16). Further, peptide bonds involving Aib residues should be less susceptible to enzymatic cleavage, resulting in analogs with long lasting *in vivo* activity (17).

MATERIALS AND METHODS

All peptides were synthesized by standard solution phase procedures, using dicyclohexylcarbodiimide (DCC) or DCC-1-hydroxybenzotriazole mediated couplings in CH_2Cl_2 or dimethylformamide (18). Boc groups and methyl esters were used for amino and carboxyl protection, respectively. Removal was effected with HCl/tetrahydrofuran for Boc groups and saponification in 2 N NaOH- CH_3OH for the esters. Analogs with Aib residues were prepared by 2 + 3 condensation, while Gly-Gly analogs were prepared by 1 + 4 coupling. Peptides were checked for homogeneity by t.l.c. on silica gel using 9:1 CHCl_3 - CH_3OH or 4:1:1 n-butanol-acetic acid-water systems. Peptide structures were also characterized by 270 MHz ^1H and 67.89 MHz ^{13}C n.m.r. spectra. The amino acid compositions determined for the analogs are presented in Table 1.

Spectroscopic measurements

All n.m.r. spectra were recorded on a Bruker WH-270 FT-NMR spectrometer at the Bangalore NMR Facility. The deuterium resonance of the solvent served as the internal field frequency lock. All chemical shifts are expressed as δ (p.p.m.) downfield from internal TMS. Assignment of resonances was done using spin de-

coupling and solvent and temperature variation studies. Peptide concentrations were ~ 10 mg/ml.

I.r. spectra were recorded on a Perkin Elmer 580 spectrometer, in CHCl_3 solution, using a pathlength of 2 mm. Peptide concentrations were ~ 1.5 – 2 mM.

In vivo biological activity

The enkephalin analogs were tested as described earlier, by monitoring behavioral effects in mice following intracerebral administration of the peptides (19).

RESULTS AND DISCUSSION

Assignment of NH resonances

In all the peptides studied, NH resonances of Phe and Met were unambiguously identified by spin decoupling experiments, which established the connections between C^βH_2 , C^αH and NH protons. The assignment of the Phe C^βH_2 ($\sim 3.0\delta$) and Met C^βH_2 ($\sim 1.9\delta$) protons is unambiguous. The lone triplet is readily assigned to Gly NH. The Aib NH singlets were distinguished from the C-terminal carboxamide singlets, on the basis of broadening of the latter at elevated temperatures. An unequivocal assignment of the two doublets to Phe and Met NH groups could not be made for the peptide Tyr-Aib-Aib-Phe-Met- NH_2 on the basis of decoupling experiments as their C^αH resonances overlap at 4.25δ . However, as discussed later, ambiguity in assignments does not negate the conformational conclusions. The chemical shifts of NH resonances in enkephalin analogs and protected tetrapeptides are summarized in Tables 2 and 3, respectively.

TABLE I
Amino acid analysis^a of enkephalin analogs

Peptide	Tyr	Gly	Phe	Met/Leu
Tyr-Gly-Gly-Phe-Met- NH_2	1.0	1.88	1.13	1.13
Tyr-Gly-Gly-Phe-Leu- NH_2	1.02	1.84	1.0	1.08
Tyr-Aib-Gly-Phe-Met- NH_2	0.98	1.15	1.0	1.08
Tyr-Gly-Aib-Phe-Met- NH_2	0.92	1.08	1.14	1.0
Tyr-Aib-Aib-Phe-Met- NH_2	0.94	—	1.0	0.82

^aAib was not analysed because of its low ninhydrin colour value.

Delineation of intramolecular hydrogen bonds

Enkephalin analogs. The temperature dependence of NH chemical shifts was determined in $(\text{CD}_3)_2\text{SO}$ and the temperature coefficients ($d\delta/dT$) are summarized in Table 2. The high $d\delta/dT$ values ($> 3.5 \times 10^{-3}$ p.p.m./ $^\circ\text{C}$) observed for Phe and Met/Leu NH groups in Tyr-Gly-Gly-Phe-Met/Leu-NH₂ indicate that they are exposed to the solvent. These results are in agreement with values reported for the cationic form of Met-enkephalin (7, 20) and Met-enkephalinamide (20).

The low $d\delta/dT$ values observed for Phe NH in Tyr-Aib-Gly-Phe-Met-NH₂ indicate that it is involved in an intramolecular hydrogen bond. The tendency of Aib residues to initiate the formation of β -turns, incorporating 4 \rightarrow 1 hydrogen bonds in organic solvents, is well established (13, 16). For Tyr-Aib-Gly-Phe-Met-NH₂, a β -turn conformation with Aib² and Gly³ residues and a 4 \rightarrow 1 hydrogen bond between Phe NH and Tyr CO groups (Fig. 1a) is compatible with the n.m.r. results and also with the known stereochemical preferences of Aib residues. Aib residues are stereochemically restricted to conformations in the region $\phi \sim \pm 60 \pm 20^\circ$ and $\psi \sim \pm 30 \pm 20^\circ$ (13). Hence the proposed β -turn conformation must be either Type III (III') or Type I (I')(21). The enantiomeric β -turns are considered since both corner residues are achiral. The difference between the Type I (I') and Type III (III') conformations is only a small change in the ϕ , ψ values of the $i+2$ (Gly³) residue (Type I $\phi_{i+2} \sim -90^\circ$, $\psi_{i+2} \sim 0^\circ$; Type III $\phi_{i+2} \sim -60^\circ$, $\psi_{i+2} \sim -30^\circ$).

In Tyr-Gly-Aib-Phe-Met-NH₂, the Phe NH

has a high $d\delta/dT$ value, whereas Met NH yielded a very low $d\delta/dT$ value, clearly indicating the involvement of Met NH in an intramolecular hydrogen bond. The n.m.r. data thus favors a conformation with an Aib³-Phe⁴ β -turn (Fig. 1b), stabilized by a 4 \rightarrow 1 hydrogen bond between the Gly CO and Met NH groups. The presence of L-Phe at position $i+2$ in the β -turn would favor a Type III or Type I structure.

In Tyr-Aib-Aib-Phe-Met-NH₂ both Phe and Met NH groups show very low $d\delta/dT$ values in contrast to the lone observed Aib NH group, which has a high $d\delta/dT$ value. While in this peptide, unambiguous assignment of the Phe and Met NH groups could not be made because of overlap of C $^\alpha$ H resonances, their identical temperature dependences permit definitive conformational interpretations. The involvement of Phe and Met NH groups in intramolecular hydrogen bonds is inferred from the n.m.r. results. A consecutive β -turn conformation, comprised of two successive Type III or Type III-Type I turns (Fig. 2) is compatible with the n.m.r. data. The proposed structure is consistent with the tendency of Aib-X sequences to form Type III (I) β -turns.

Protected tetrapeptides

The enkephalin analogs described above have only very limited solubility in apolar organic solvents, like chloroform. Hence conformational studies were restricted to polar, non-aqueous systems like $(\text{CD}_3)_2\text{SO}$. In order to substantiate the conclusions derived from n.m.r. studies on the enkephalin analogs, the corresponding Boc protected tetrapeptide amides were examined. Since in the β -turn conformations discussed

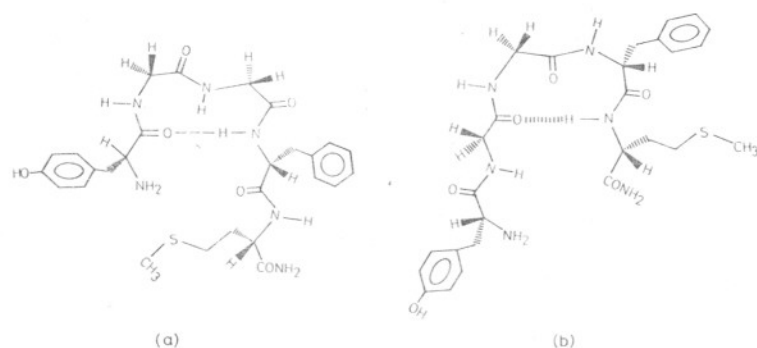


FIGURE 1

β -turn conformations considered for enkephalins (a) β -turn with residues 2 and 3 at the corners (b) β -turn with residues 3 and 4 at the corners.

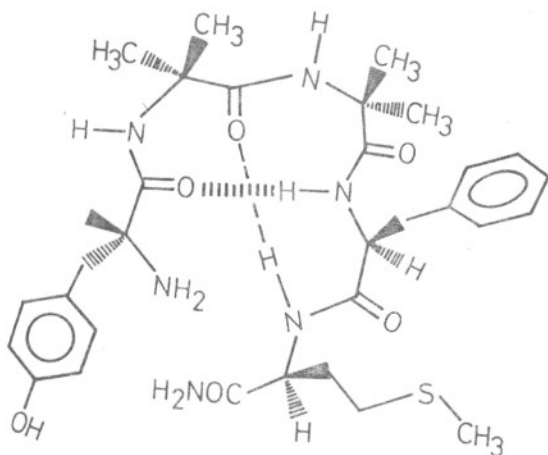


FIGURE 2

Proposed incipient 3_{10} helical conformation for Tyr-Aib-Phe-Met-NH₂. Consecutive β -turns with Aib²-Aib³ and Aib³-Phe⁴ as the corner residues are stabilized by intramolecular 4 \rightarrow 1 hydrogen bonds.

above, only the CO group of Tyr¹ is essential, the protected tetrapeptides may be considered as formally analogous to the enkephalin analogs, insofar as backbone folding is considered. The higher solubility of the Boc peptides permits the use of i.r. studies in CHCl₃ (22) and n.m.r. solvent titration experiments (23) to detect hydrogen bonded structures.

The $d\delta/dT$ values for the NH groups in the various tetrapeptides are summarized in Table 3. In Boc-Gly-Gly-Phe-Met-NH₂ all the NH groups appear solvent exposed ($> 4 \times 10^{-3}$ p.p.m./ $^{\circ}$ C), whereas in Boc-Aib-Gly-Phe-Met-NH₂ Phe NH is shielded from the solvent. In Boc-Gly-Aib-Phe-Met-NH₂ and Boc-Aib-Aib-Phe-Met-NH₂

both Phe and Met NH groups yield low $d\delta/dT$ values, characteristic of intramolecularly hydrogen bonded NH groups. For Boc-Aib-Gly-Phe-Met-NH₂ a Aib-Gly β -turn, with a 4 \rightarrow 1 hydrogen bond between Boc CO and Phe NH is consistent with the data. However, the relative insensitivity of the Met NH chemical shift to solvent composition (Fig. 3) and its moderate $d\delta/dT$ value in (CD₃)₂SO (3.7×10^{-3} p.p.m./ $^{\circ}$ C), suggests that further folding of the backbone as a Gly-Phe β -turn may occur. This feature is probably weakened in a polar solvent like (CD₃)₂SO (24).

In Boc-Aib-Aib-Phe-Met-NH₂ the n.m.r. data is consistent with an incipient 3_{10} helical structure having two intramolecular 4 \rightarrow 1 hydrogen bonds (Boc CO---HN Phe and Aib¹ CO---HN Met). The proposed conformation is compatible with the low $d\delta/dT$ values and insensitivity of Phe and Met NH chemical shifts to solvent composition in a CDCl₃-(CD₃)₂SO system (Fig. 3). The n.m.r. data for Boc-Gly-Aib-Phe-Met-NH₂, support a similar consecutive β -turn structure. In all of the above three protected tetrapeptides clear evidence for the occurrence of intramolecular hydrogen bonded structures is obtained from i.r. studies in dilute CHCl₃ solution, where ν_{NH} bands are observed at ~ 3320 cm⁻¹ (Fig. 4). By contrast, in Boc-Gly-Gly-Phe-Met-OMe only the ν_{NH} (free) band is observed at 3430 cm⁻¹.

In the present study the effect of peptide aggregation on the conformation and consequently the n.m.r. parameters has not been explicitly considered. This is reasonable since earlier studies from this laboratory on Aib containing oligopeptides (25-28) suggest that

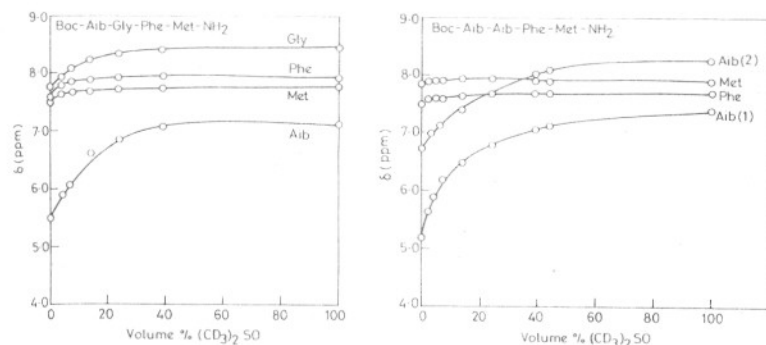


FIGURE 3

Solvent dependence of NH chemical shifts for the peptides Boc-Aib-Gly-Phe-Met-NH₂ and Boc-Aib-Aib-Phe-Met-NH₂ in CDCl₃-(CD₃)₂SO mixtures of varying composition.

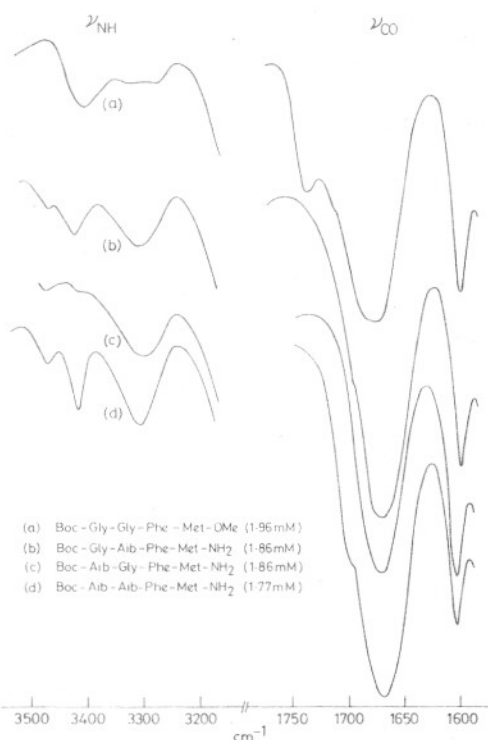


FIGURE 4

I.r. spectra of Boc protected tetrapeptides in CHCl_3 solution.

aggregation is insignificant in $(\text{CD}_3)_2\text{SO}$ at the concentrations used. A similar conclusion has been reached for the Aib analogs of bradykinin (29). Association of peptides is important in chloroform at the concentrations employed in n.m.r. studies (30). However, for the conformationally constrained Aib peptides aggregation appears to occur without significant disruption of folded structures (25–28). In the present study solvent titration experiments in CDCl_3 - $(\text{CD}_3)_2\text{SO}$ mixtures have been used as a diagnostic for shielded NH groups. The agreement between these results and the temperature dependence studies in $(\text{CD}_3)_2\text{SO}$ suggests that association, if any, in CDCl_3 does not vitiate the conclusions. The excellent agreement obtained between i.r., n.m.r. and X-ray diffraction studies of Aib peptides (13) lends further support to the conformational conclusions drawn above. Finally, in the case of Boc-Aib-Aib-Phe-Met-NH₂, a single crystal

X-ray diffraction study has indeed confirmed that the proposed consecutive β -turn structure does occur in the solid state (31).

The studies summarized above on the protected tetrapeptides establish that Boc-Aib-Gly-Phe-Met-NH₂ and Boc-Aib-Aib-Phe-Met-NH₂ favour conformations similar to those suggested for the corresponding enkephalin analogs. In the case of Boc-Gly-Aib-Phe-Met-NH₂ an additional Gly-Aib β -turn appears to be stabilized, as compared to Tyr-Gly-Aib-Phe-Met-NH₂, in which only the Aib-Phe β -turn is supported by the data. The reasons for this difference remain obscure. The present results suggest that replacement of Gly residues in enkephalins and related peptides by Aib residues results in the generation of folded backbone conformations. An earlier report has provided evidence for the role of Aib residues in imparting stereochemical rigidity in enkephalins, as monitored by circular dichroism (32).

Correlation with biological activity

The *in vivo* activity of the enkephalin analogs reported in Table 4 indicates that Tyr-Aib-Gly-Phe-Met-NH₂ and Tyr-Aib-Aib-Phe-Met-NH₂ exhibit considerably higher activity than Met-enkephalin-amide or the Gly²-Aib³ analog. The high activity of Tyr-Aib-Gly-Phe-Met-NH₂ suggests that the receptor bound conformations are likely to incorporate either a Type III (I) or Type III' (I') β -turn, centred at residues 2 and 3. In view of the stabilization of β -turns by Aib residues, it is unlikely that this structural feature will be broken on receptor binding. The high biological activity observed for D-Ala² enkephalin analogs (33, 34) and the positive ϕ , ψ values determined for the Gly residue in the crystal structure of Leu-enkephalin (9), lead us to speculate that the "active conformation" of Tyr-Aib-Gly-Phe-Met-NH₂ possesses an Aib²-Gly³ Type III' (I') β -turn. Thus, the results of the present study suggest that conformations with a β -turn at positions 2 and 3 permit the appropriate orientation of the Tyr¹ and Phe⁴ residues at the receptor site (35). The long lasting *in vivo* biological activity of the Aib² analog may also partly reflect the resistance of this peptide to enzymatic degradation. However, activity may not be expressed

TABLE 4
In vivo biological activity^a and backbone conformation of enkephalin analogs

Peptide	Dose (μ g)	Mean recovery time (min)	β -turn position
Control (1:4 alcohol-water)	--	5 \pm 1	--
Tyr-Gly-Gly-Phe-Met-NH ₂	100	51 \pm 20	--
Tyr-Gly-Gly-Phe-Leu-NH ₂	100	38 \pm 12	--
Tyr-Aib-Gly-Phe-Met-NH ₂	50	128 \pm 26	Aib ² -Gly ³
Tyr-Aib-Aib-Phe-Met-NH ₂	50	154 \pm 32	Aib ² -Aib ³
			Aib ³ -Phe ⁴
Tyr-Gly-Aib-Phe-Met-NH ₂	100	28 \pm 6	Aib ³ -Phe ⁴

a) *In vivo* biological activity was determined using a behavioural assay in mice as described in Ref. 19. Recovery times are averaged over groups of 15–20 mice. The reported values differ slightly from Ref. 19 due to the larger number of mice used.

unless the Aib²-Gly³ β -turn is a structural feature capable of recognition by the receptor.

Of the three Aib analogs, Tyr-Gly-Aib-Phe-Met-NH₂ exhibits the lowest *in vivo* activity. This suggests that the Aib³-Phe⁴ β -turn results in a conformation which does not permit optimal interactions at the receptor. Further, *in vivo* degradation by cleavage of the Tyr¹-Gly² bond may diminish activity (36).

Tyr-Aib-Aib-Phe-Met-NH₂, which adopts a consecutive β -turn structure, exhibits the highest *in vivo* activity. The presence of two Aib residues in this analog considerably reduces backbone conformational flexibility, suggesting that receptor interactions are probably mediated through the observed solution conformation. The "active conformation" in this case is presumably an incipient 3_{10} helical structure (Fig. 2). It is intriguing that a helical structure was proposed for enkephalins several years ago by Goldstein and coworkers (37) but has not found favour in the literature.

In the present study, the introduction of stereochemical constraints has been used as a filter for narrowing the range of backbone conformations, which may result in biological activity. While the highly active analogs possess β -turns centred at positions 2 and 3, this conformational feature may not be an essential requirement for receptor binding. For example exceptional activity has been observed with *N*-methyl Phe⁴ analogs, where the Tyr¹ CO---HN Phe⁴ hydrogen bond is absent (38, 39). Double bond isosteres, where the

Tyr¹-Gly² peptide bond is replaced by an olefinic linkage, are also active (40), as are cyclic analogs with limited conformational possibilities (41). However, in these cases also, folded geometries closely resembling the β -turn structures may be generated. The occurrence of multiple receptor sites *in vivo* and the problems of side-chain flexibility provide further hurdles to be overcome before more definitive conformation-function correlations can be drawn.

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