Articles

Design, Synthesis, and Binding Characteristics of an Opiate Receptor Mimetic Peptide†

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An artificial tetracontapeptide that mimicked the recognition/binding properties of naturally occurring opioid receptors was designed, synthesized, and purified to homogeneity. The design of the primary structure of the receptor mimetic peptide (RMP) accounted for secondary structure prediction rules and for the stereochemical anatomy of various enkephalin and morphine derivatives. The affinity of a series of opioid and nonopioid peptides to RMP was determined from their potency in displacing the binding of enzymatically prepared (^{14}C)-[Leu]-enkephalin. The competition studies revealed that the binding is specific for endogenous opiate peptides, stereoselective for the naturally occurring L isomer of [Leu]-enkephalin, and discriminative for closely related opioid peptides. The thermodynamic parameters associated with the binding of [Leu]-enkephalin to RMP were evaluated from equilibrium studies at different temperatures. The van't Hoff plot of the resulting data was curvilinear. The formation of the ligand–RMP complex was characterized by a decrease both in entropy and in enthalpy with temperature. The thermodynamic behavior provided some evidence that hydrophobic interactions played a prominent role in stabilizing the [Leu]-enkephalin–RMP complex.

In recent years much attention has been focused on the possible molecular architecture of opiate receptors. The most promising strategy to gain a deeper insight into the receptors' nature would be one that enables us to elucidate its structural organization by physical and chemical methods. An indispensable prerequisite for these analytical procedures is the isolation of the receptor in a highly purified form. However, it is an intriguing and often frustrating problem to solubilize the opiate receptor in an active form from the surrounding lipid bilayer. Recently, several reports have appeared describing the successful isolation, purification, and preliminary characterization of opiate receptors,[1-4] but there is still little direct insight into its molecular nature. Nevertheless, several interesting features of the opiate receptor have been revealed. The molecule has a mass in excess of 10^5 daltons.[3,4] It is composed of several subunits[4] and displays a Stokes radius of 70 Å.[3] The predominantly proteinaceous character of opiate receptors is well established,[3,5,6] but there is also some evidence that the receptor molecule contains lipidic[6] and glycosidic components.[7]

A quite different method of characterizing the opiate receptor is based on the assumption that an efficient receptor is topologically complementary to the stereochemical nature of its ligand. The so-called "lock and key" concept proposed in 1894 by Emil Fischer[8] is generally accepted as valid to the present day. If a large number of differently shaped keys are available that all fit the lock, then the three-dimensional shape of the lock may be inferred from some structural features common to all keys. A series of models of the geometry of the opiate receptor's binding area has been derived from the molecular structure of a variety of opioid pharmacophores by using this approach.[9-18]

In this work we want to present a completely different method in order to shed some light upon the molecular identity of the opiate receptor. An artificial tetracontapeptide was designed with an amino acid sequence (Figure 1a) based upon theoretical studies on model receptors, experimental data of biologically active receptors, and studies on structure–activity relationships of a variety of opiate drugs. In addition, we took into account prediction rules describing the formation of secondary structural elements as a function of the amino acid sequence. The

† Abbreviations used are: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Z, benzyloxycarbonyl; Pht, phthaloyl; OMe, methyl ester; Ph, phenyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, dimethylformamide; TFA, trifluoroacetic acid; MSH, melanocyte-stimulating hormone.

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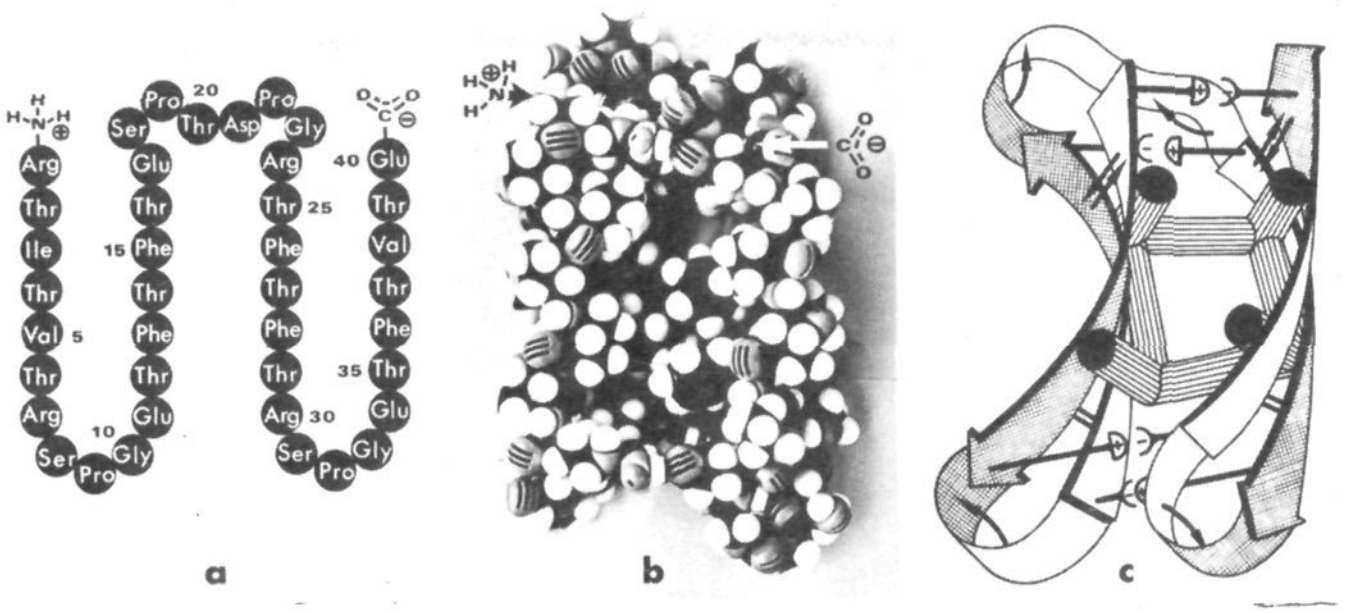

Figure 1. (a) Amino acid sequence of the receptor mimetic peptide (RMP). (b) Structural diagram of the proposed three-dimensional shape of RMP. Thick arrows depict β-sheet strands, thin arrows indicate hydrogen bonds, thin lines denote salt bridges, and hydrophobic clusters are given as shaded cylinders. (c) Space-filling model of the proposed three-dimensional structure of RMP.

three-dimensional arrangements were controlled by the construction of (CPK) space-filling models, in particular, the "fit" of several opioid ligands in the spatial structure of the "receptor mimetic peptide" (RMP). RMP was synthesized in order to explore its capacity of mimicking the primary functions of natural opioid receptors: recognition and binding of opiate ligands. The analysis of the binding characteristics of RMP should provide a working model of the molecular interactions between opiates and biological opioid receptors. A similar approach was recently reported by Gutte et al.,[19] who prepared an artificial peptide with nuclease-like activities.

Rationale of RMP's Primary Structure. RMP is composed of 40 amino acid residues. Thus, its size roughly corresponds to 1% of the size of naturally occurring opiate receptors. Accordingly, the tetracontapeptide fulfills solely the minimal structural requirements for the binding area and its immediate surroundings. The molecular architecture of RMP (Figure 1b,c) resembles a twisted "rectangular" trough, which enables the binding of ligands in the absence of bulk water. The side walls of the trough are made up of antiparallel β-pleated sheets, each formed by two β-strands that are linked by a reverse turn. The connecting structure between the two β-pleated sheets is made up by two partially overlapping β-turns, which form an enlarged loop structure where the peptide chain folds back by nearly 180°. The characteristic curvature of the RMP topography is caused by the β-sheet structures, most of which are known to display a pronounced twist.[20] The front wall is supplied by the side chains of glutamic acid and arginine residues, which form two salt bridges. The same holds true for the back wall, an additional structural element of which is the above-mentioned loop.

In order to realize the secondary structural elements postulated above, the primary structure of RMP was devised as follows: the segments comprising amino acid residues 2 to 6, 12 to 16, 25 to 29, and 35 to 39 each constitute a series of alternating hydrophilic and hydrophobic residues. The tendency of these kinds of polymers to adopt β-conformations has been shown by Brack and Caille.[21] According to secondary structure prediction rules,[22] the β-sheet potential of these segments ranges from 1.26 to 1.33. Polar and nonpolar side chains are directed toward the exterior and the interior, respectively, of RMP. Six hydrogen bonds cross-link each double-stranded antiparallel structure. The threonine residues 2, 4, 6, 25, 27, and 29 are twofold hydrogen bonded in reverse directions to the threonine residues 16, 14, 12, 39, 37, and 35, respectively. The β-pleated sheet arrangement is further stabilized by hydrophobic and/or π–π interactions between the apolar side chains of Ile-3 and Phe-15, Val-5 and Phe-13, Phe-26 and Val-38, and Phe-28 and Phe-36. β-Turn structures in RMP are considered to occur at residues 8–11, 18–21, 21–24, and 31–34. The β-turn potential[22] of these tetrapeptides was determined to be 1.37–1.39. The peptide chain region comprising residues 18 through 24 is believed to be composed of two adjacent β-turn-like structures that have residue 21 in common. This overlap is thought to prevent the "incomplete β-turns" from folding back through 180° and therefore to enable the emergence of an extended loop structure, which then constitutes a full chain reversal. Similar arrangements have been described for the *lac* repressor[23] and several protease inhibitors.[22] The reverse turns comprising residues 8 to 11 and 31 to 34 are stabilized by hydrogen bonds between the α-carbonyl moieties of Ser-8 and Ser-31 and the α-imino groups of Glu-11 and Glu-34, respectively. The two antiparallel β-pleated sheets forming the side walls of the model receptor are tied together in a nearly parallel arrangement not only by the extended loop (residues 18–24) but also by four salt bridges formed by glutamic acid and arginine side chains: Arg-1 to Glu-40, Arg-7 to Glu-34, Glu-11 to Arg-30, and Glu-17 to Arg-24. The arginine–glutamate pairs were chosen due to their stability and highly directional character. These properties result from the cooperative resonance of the guanidine–carboxylate double ionic bonds.[24] Additional binding elements are given by π–π interactions between phenylalanine residues 13, 15, 26, and 28. Their bulky benzyl groups also provide for the floor of the trough.

To determine the essential chemical functions and their correct spatial disposition in the binding region of RMP, we referred to studies on structure–activity relationships of morphine and enkephalin derivatives. Morphine- and oripavine-related structures that possess a relatively rigid framework are assumed to be topographically analogous

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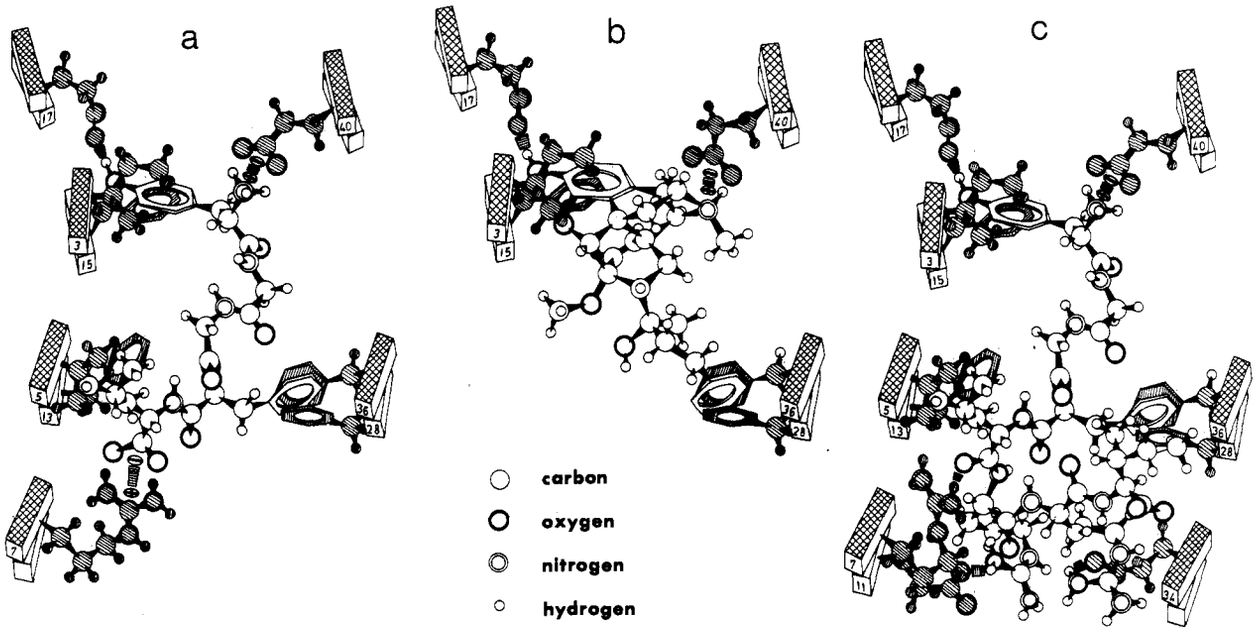

Figure 2. Stereoviews of the proposed molecular structure of the (a) [Leu]-enkephalin–RMP complex, (b) the PEO–RMP complex, and the (c) dynorphin$_{1-8}$–RMP complex. Amino acid side chains of RMP are indicated by shading. Their position is denoted on the front of the bars, which in turn epitomize segments of RMP's backbone.

to [Met]-enkephalin.[25] Therefore, the stereochemical anatomy of the enkephalins, dynorphin$_{1-8}$, des-Gly$_3$-[Leu]-enkephalin, *all*-D-[Leu]-enkephalin, morphine, and oripavine derivatives were used as steric probes to evaluate the spatial arrangement of the critical binding sites by means of model building. The theories of the molecular structure of the opioid receptor usually assume both an anionic and a lipophilic binding site for electrostatic and hydrophobic interactions within the tertiary amino group and with ring A, respectively, in the tyramine moiety of opiates. The tyramine portion present in opiates and enkephalins is considered to be functionally equivalent in the recognition and binding process at opioid receptors.[26,27] According to space-filling models, RMP provides for the postulated lipophilic binding site via the side chains of residues Ile-3 and Phe-15. The anionic binding area is represented by the γ-carboxylate function of Glu-40, which can form a salt bridge with the positively charged tertiary amine of morphine derivatives or with the α-amino group of opiate peptides. This newly emerged, intermolecular bond at least partially replaces the preexisting guanidine–carboxylate double ionic bond (see above). The reductions in morphinomimetic potencies provoked by modification of the C-3 hydroxy group of morphine derivatives[28] and the phenolic hydroxy group of [Met]-enkephalin[25] parallel each other. Probably, these hydroxy groups, which are essential for biological activity,[29,30] correspond to each other. Although the precise mode of interaction of the hydroxy groups with the receptor is still unresolved, a hydrogen bonding seems quite reasonable. An opportunity to establish this hydrogen bond is provided by the γ-carboxylate function of Glu-17 of RMP. A second lipophilic binding site, roughly 10 Å apart from the first one (see above), was proposed by Bentley and Lewis[13] on account of structure–activity studies on oripavine derivatives. The addition of a phenylethyl group to the C-19 atom of oripavine resulted in a pronounced increase of activity relative to morphine. Bradbury et al.[31] suggested an analogy between the phenylethyl substituent of oripavine and the benzyl moiety of phenylalanine in the enkephalins. This assumption was supported by the finding that the phenol–phenyl distance in [Leu]-enkephalin[32,33] and the phenol–indol separation in [Trp$_4$,Met]-enkephalin[34] closely resembled the intramolecular distance between the centers of the two aromatic rings in the above-mentioned oripavine derivative. Therefore, a second lipophilic binding site on RMP comprising the phenylalanine residues at positions 28 and 36 was designed. Removal of the fifth residue of [Leu]- or [Met]-enkephalin results in a tetrapeptide that displays significant, albeit weak, activities.[29,34,35] Furthermore, contraction of the side-chain length of the fifth residue reduces its hydrophobicity and results in a decrease of binding affinity.[29] These findings are indicative of a third lipophilic binding site. The 5th and 13th positions of RMP, valine and phenylalanine, may provide for this additional binding site. Removal of the C-terminal carboxyl group of the enkephalins leads to weakly active analogues.[35,36] Presumably, the importance

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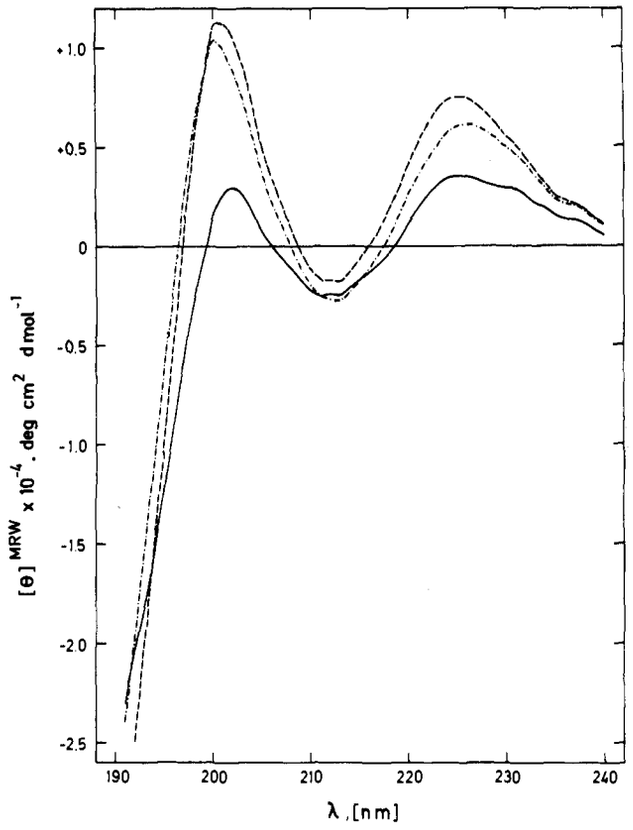

Figure 3. CD spectra of RMP in the far UV region: (—) observed in 0.01 M phosphate buffer, pH 7. Calculated on the basis of standard spectral data[61] for the secondary structure pattern as predicted according to Chou and Fasman:[22] (-•-), 70% β-sheet and 30% β-turn; (- - -) 62.5% β-sheet and 37.5% β-turn.

of the α-carboxyl moiety depends on electrostatic interactions with a polar region on the receptor. The δ-guanidine group of Arg-7 of RMP may represent such a polar site capable of interacting with the ligand's C-terminal carboxyl group via hydrogen or ionic bonding. The postulated fit of [Leu]-enkephalin and of 7α-[1(*R*)-hydroxy-1-methyl-3-phenylpropyl]-6,14-*endo*-ethenotetrahydrooripavine (PEO) into RMP is illustrated in Figure 2a,b. Besides the enkephalins, dynorphin$_{1-8}$,[37,38] the N-terminal pentapeptide of which corresponds to the amino acid sequence of [Leu]-enkephalin, was selected to model RMP. Relative to [Leu]-enkephalin, this octapeptide may additionally bind to RMP via electrostatic and/or hydrophobic interactions as illustrated in Figure 2c. The des-Gly$_3$ analogue of [Leu]-enkephalin is very weakly active.[39] As observed by model building, the truncated peptide could not simultaneously bind to both the second and the third lipophilic site. An *all*-D-[Leu]-enkephalin is less effective by a factor of ~200 relative to its L enantiomer.[40] Stereochemical studies using CPK models revealed that there exist several conceivable conformers of the *all*-D isomer, which could partially exploit the binding facilities supplied by RMP. Under the optimal conditions, three

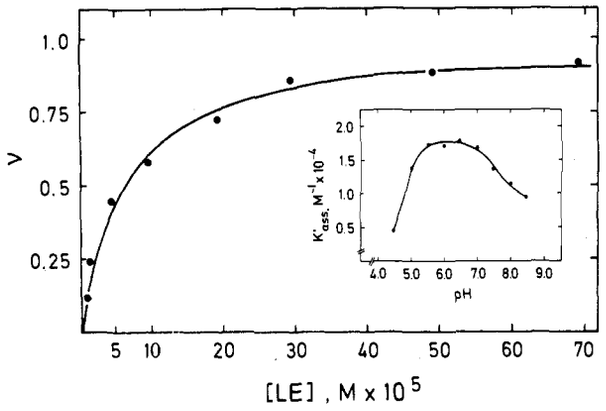

Figure 4. Binding of (*3-Gly*-1-^{14}C)-[Leu]-enkephalin to RMP. Experimental data are expressed as ν, the moles of [Leu]-enkephalin bound per mole of RMP. ν is plotted as a function of free [Leu]-enkephalin concentration [LE]. Equilibrium dialysis was carried out as described in the Experimental Section. RMP was present at 1×10^{-5} M. Dialysis time at 5 °C was 48 h. Inset: pH dependence of [Leu]-enkephalin binding to RMP. The values of $K_{assoc}' = 1/K_D'$ were derived from Scatchard analyses of equilibrium dialyses. Apart from the buffer used, McIlvain's citrate (0.1 M)/disodium phosphate (0.2 M), the experiments were performed as described above.

out of six available binding sites could be occupied.

Results

CD Spectroscopy. The observed and two calculated UV circular dichroism spectra of RMP are shown in Figure 3. The experimental CD spectrum, which nearly coincides with that representing the least-squares fit (not shown), reflects the following secondary structure composition of RMP: 0% (0 residues) α-helix, 50% (20 residues) β-structure, 30% (12 residues) β-turn, and 20% (8 residues) aperiodic form. The calculated CD spectra are based on the assumption that RMP is composed of either 70% β-structure and 30% β-turn or 62.5% β-structure and 37.5% β-turn. The quantification of the structural elements was accomplished by the Chou and Fasman predictive rules.[22]

Equilibrium Dialysis Studies. (*3-Gly*-1-^{14}C)-[Leu]-enkephalin Binding to RMP. Figure 4 shows the saturation curve of the binding to RMP of [Leu]-enkephalin. When the amount of opiate ligand bound to the receptor was recorded as a function of free ligand concentration, the resulting plot illustrates that the binding followed a simple Langmuir isotherm. A plot of the same experimental data according to Scatchard[41] (Figure 5) yielded a straight line, the intercept of which with the abscissa gave a value of 0.98 ± 0.05 for the moles of ligand bound per mole of receptor. This result suggests a single binding site on RMP. The apparent dissociation constant K_D' as derived from the slope of the Scatchard plot (fitted by least squares) was 5.81 (±0.24) × 10^{-5} M (5 °C). The pH dependence of [Leu]-enkephalin binding to RMP is illustrated in the inset to Figure 4. Under otherwise identical conditions of equilibrium dialysis, (^{14}C)-[Leu]-enkephalin (10^{-3} M) had no measurable affinity to commercial glucagon (10^{-5} M) or to a synthetic proinsulin analogue (10^{-5} M).

Equilibrium Studies via UV Difference Spectroscopy. Biological macromolecules usually contain a variety of intrinsic chromophores. Changes in the environment of a chromophore, if detectable in terms of shifts in absorbance maxima and intensities, may be exploited to

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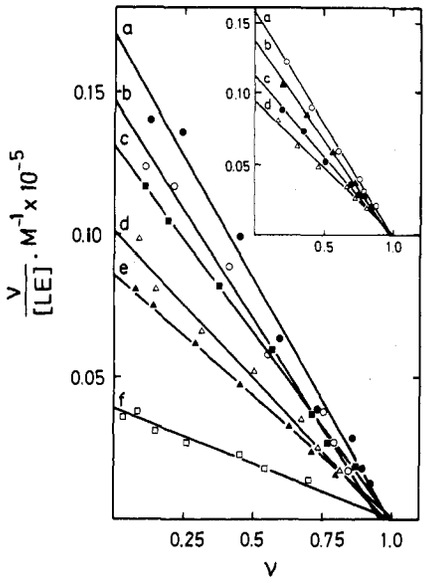

Figure 5. Scatchard plots of the binding data shown in Figure 4 and of competitive binding in the presence of enkephalin-related peptides and naloxone. (The experimental conditions were the same as described in Figure 4.) Binding of [Leu]-enkephalin (a) in the absence of competing ligands (●) and in the presence of (b) *all*-D-[Leu]-enkephalin (5×10^{-5} M) (○), (c) des-Gly_3-[Leu]-enkephalin (5×10^{-5} M) (■), (d) naloxone (5×10^{-3} M) (△), (e) [Met]-enkephalin (5×10^{-5} M) (▲), and (f) $dynorphin_{1-8}$ (5×10^{-5} M) (□). Inset: (a) des-NH_2-[Phe_1,Leu]-enkephalin (5×10^{-5} M) (○), (b) des-NH_2-[Leu]-enkephalin (5×10^{-5} M) (▲), (c) [Phe_1,Leu]-enkephalin (5×10^{-5} M) (△), (d) H-His-Phe-Arg-Trp-OH (5×10^{-3} M) (●).

monitor ligand binding in the vicinity of a chromophore.

The present binding studies took advantage of the finding that the interaction of [Leu]-enkephalin with RMP provoked significant spectral changes in the near-UV range. The red-shifted difference spectra were characterized by positive peaks above 270 nm and a series of alternating positive and negative peaks between 250 and 270 nm. The observed change in absorbancy at 260 and 284 nm was used to calculate the dissociation constants (K_D') according to the relationship[42] shown in eq 1, where

$$\frac{[LE^\circ \times RMP^\circ]}{\Delta A} = \frac{[LE^\circ + RMP^\circ]}{\Delta \epsilon} + \frac{K_D'}{\Delta \epsilon} \quad (1)$$

LE° and RMP° represent the total concentrations of [Leu]-enkephalin and of RMP, whereas the absorbancy change and the respective change of the molar absorptivity are indicated by ΔA and $\Delta \epsilon$. The experimental data of $[LE^\circ \times RMP^\circ]/\Delta A$ were plotted against $[LE^\circ + RMP^\circ]$ for four temperatures and two wavelengths where the absorbancy changes were most pronounced. A typical diagram is shown in Figure 6. The graphs were linear within the experimental errors. The reciprocal slope yielded $\Delta \epsilon$. $K_D'/\Delta \epsilon$ could be obtained as the intercept of the straight line with the ordinate.

The apparent dissociation constants (K_D') for [Leu]-enkephalin bound to RMP were as follows: at 15 °C, 7.04 (±0.80) × 10^{-5} (284 nm) and 6.85 (±0.65) × 10^{-5} M (260 nm); at 20 °C, 7.52 (±0.55) × 10^{-5} M (284 nm) and 7.87 (±0.48) × 10^{-5} M (260 nm); at 30 °C, 9.80 (±0.85) × 10^{-5} (284 nm) and 9.24 (±0.70) × 10^{-5} M (260 nm); at 35 °C, 1.19 (±0.10) × 10^{-4} (284 nm) and 1.25 (±0.20) × 10^{-4} m (260 nm).

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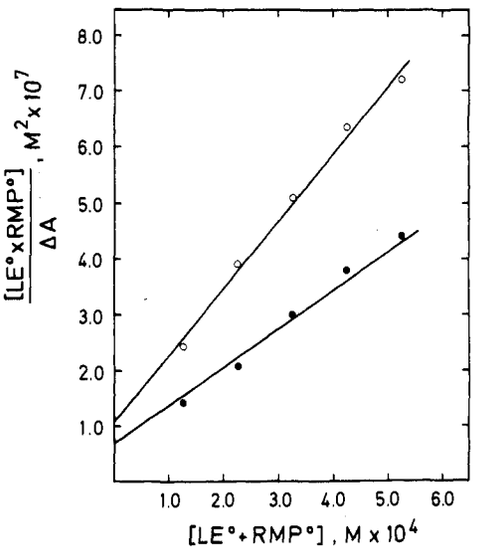

Figure 6. Binding of [Leu]-enkephalin to RMP. The experimental data were plotted as $(LE^\circ \times RMP^\circ)/\Delta A$ vs. $(LE^\circ + RMP^\circ)$. The values of ΔA were determined as described in the Experimental Section. RMP was present at 2.5×10^{-5} M. Absorbancy changes were measured at 30 °C, 260 nm (○), and at 30 °C, 284 nm (●).

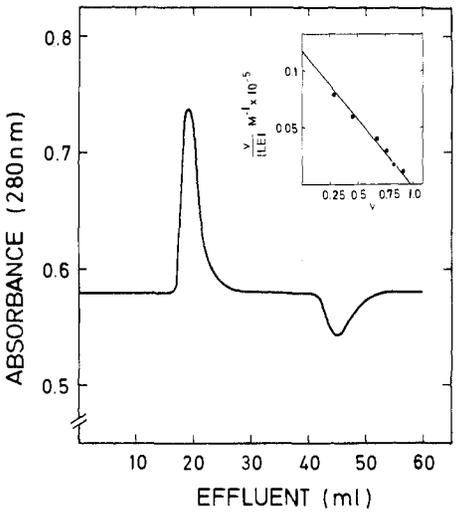

Figure 7. Representative elution pattern for the measurement of [Leu]-enkephalin binding to RMP via gel filtration. The concentration of [Leu]-enkephalin was 5×10^{-4} M; the amount of RMP was 4.46 mg. The temperature was 25 °C. Gel filtration was performed as described in the Experimental Section. Inset: The experimental data appear as a Scatchard plot. The concentration of the ligand ranged from 5×10^{-5} to 10×10^{-4} M. The amount of RMP was 4.46 mg for each run.

Characterization of Ligand–RMP Interactions by Gel Filtration. A representative elution profile for the [Leu]-enkephalin–RMP system is depicted in Figure 7. The amount of bound ligand was calculated from the area of the negative peaks obtained during a given run. The resulting data were plotted in a Scatchard graph (Figure 7), which displayed a straight line intercepting the abscissa at 0.97. This finding is in good agreement with that of equilibrium dialysis studies described above and indicates a single binding site. The dissociation constant (K_D') was 8.3 (±0.2) × 10^{-5} M (25 °C).

Temperature Dependence of the [Leu]-enkephalin Binding to RMP. The standard free energy change ($\Delta G^\circ{}'$) for the binding of [Leu]-enkephalin to RMP can be easily calculated from the observed equilibrium association constants ($K_{assoc}' = 1/K_D'$). The determination of

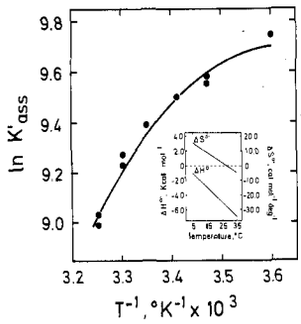

Figure 8. Van't Hoff plot for the binding to RMP of [Leu]-enkephalin. Inset: Variation of the enthalpy change ($\Delta H°'$) and entropy change ($\Delta S°'$) with temperature upon binding of [Leu]-enkephalin to RMP, calculated from eq 3 and 4.

the entropy and enthalpy changes ($\Delta S°$, $\Delta H°'$) requires knowledge of the temperature dependence of the binding constants. An analysis of ln K_{assoc}' as a function of $1/T$ yielded a nonclassical, curvilinear van't Hoff plot (Figure 8). The standard free energy changes were fitted to a series expansion in temperature.[43]

$$\Delta G°' = -RT \ln K_{assoc}' = A + BT + CT^2 \quad (2)$$

and the remaining thermodynamic parameters were determined from the appropriate derivatives,

$$\Delta H°' = A - CT^2 \quad (3)$$

$$\Delta S°' = -B - 2CT \quad (4)$$

$$\Delta C_p°' = -2CT \quad (5)$$

where $\Delta C_p°'$, is the heat-capacity (at constant pressure) change of the reaction.

As illustrated in Figure 8, the changes in both enthalpy and entropy decrease with increasing temperature. The binding process is enthalpically and entropically driven at temperatures between 5 and 28 °C. Above 28 °C, the driving force for ligand–RMP association changes to purely enthalpic. At 25 °C, the value of $\Delta C_p°'$ is −202 cal mol^{-1} deg^{-1}.

Competitive Binding of (*3-Gly*-1-^{14}C)-[Leu]-enkephalin with Various Opioid Peptides. The binding strength to RMP of several opiate peptides was determined from their potency in displacing [Leu]-enkephalin binding. A given peptide (I) was incubated in the presence of [Leu]-enkephalin, and the data obtained from equilibrium dialysis experiments were analyzed in the form of Scatchard plots (Figure 5). The dissociation constant (K_i) of the respective ligands is given by eq 6.[44] The binding

$$K_i = [I]°/(K_{D,i}'/K_D' - 1) \quad (6)$$

of [Leu]-enkephalin in the absence or presence of a fixed concentration of ligand I, [I]°, yielded K_D' and $K_{D,i}'$ as the reciprocal slope of the respective scatchard plot. The capacity to compete with (^{14}C)-[Leu]-enkephalin for binding to RMP decreased in the following order (5 °C): dynorphin$_{1-8}$ [K_i = 1.47 (±0.18) × 10^{-5} M], [Met]-enkephalin [K_i = 4.95 (±0.21) × 10^{-5} M], [Phe$_1$,Leu]-enkephalin [K_i = 9.55 (±0.36) × 10^{-5} M], des-Gly$_3$-[Leu]-enkephalin [K_i = 1.60 (±0.15) × 10^{-4} M], des-NH$_2$-[Leu]-enkephalin [K_i = 2.05 (±0.14) × 10^{-4} M], *all*-D-[Leu]-enkephalin [K_i = 4.00 (±0.22) × 10^{-4} M], des-NH$_2$-[Phe$_1$,Leu]-enkephalin [K_i = 6.20 (±0.22) × 10^{-4} M], the MSH fragment H-His-Phe-Arg-Trp-OH [K_i = 5.95 (±0.45) × 10^{-3} M], naloxone [K_i = 7.46 (±0.3) × 10^{-3} M]. Both, the C-terminal octapeptide of cholecystokinin (CCK-8) and the MSH subunits H-Tyr-Val-Met-Gly-OH, H-Asp-Arg-Phe-Gly-OH, and H-Tyr-Lys-Phe-Gly-OH, the concentration of which was finally scaled up to 10^{-2} M, had no measurable inhibitory effect on the [Leu]-enkephalin binding to RMP.

Discussion

An artificial polypeptide devised to bind opiate drugs has to provide for correctly positioned functional groups essential for ligand–receptor interactions. This requirement can only be fulfilled on the premises of suitably arranged secondary structural elements. Therefore, the design of the primary structure of RMP was strongly influenced through its capability—as predicted on the basis of statistical[22] and stereochemical methods—to adopt the desired secondary structures. In order to prove or disprove the presence of the suggested secondary structure, RMP was subjected to CD analysis (Figure 3). A quantitative evaluation of the observed CD spectrum of RMP by using standard spectral data[45] revealed that 20 amino acids can be assigned to β-sheets, 12 residues have adopted the β-turn formation, 8 residues are located in random-coil regions, and α-helical structures are absent. The originally designed architecture of RMP anticipated four β-strands, each containing seven amino acid residues. This implied that all the residues positioned at the overlapping boundaries of β-sheets and β-turns were ascribed to the former structure. Consequently, the fractions of β-structures and β-turns amounted to 70 and 30%, respectively (Figure 3). The CD analysis solely confirmed the predicted percentage of reverse turns. A second estimate assigned three of the boundary residues to β-turn regions, giving a total of four reverse turns, two of which had one residue in common. Thus, the β-sheet contribution was reduced to 62.5%, and the β-turn portion was increased to 37.5% (Figure 3). Although the second assumption presented an improved prediction of the β-structures of RMP, it did not agree with the actual outcome of 50%. The observed β-form percentage is best rationalized by the assumption that the originally devised four β-strands are each represented by a pentapeptide instead of a heptapeptide. Our first estimate anticipated 30% β-bend content. This prediction exactly agreed with the observed percentage. We tentatively ascribe the resulting 12 residues to four reverse turns in the neighborhood of four proline residues, which in this arrangement are always located in the more favored second position[22] of a given β-turn. Thus, only three instead of four residues would be involved in a chain reversal. As an alternative, the 12 residues can be attributed to three β-bends if the extended loop structure illustrated in Figure 1c is assumed to merely represent a single reversed chain.

The interpretation of thermodynamic quantities in terms of peptide–peptide interactions may be speculative, since the thermodynamic parameters observed in aqueous solutions represent a composite of various processes. Nevertheless, a qualitative description of the major factors involved in the binding process can provide useful insights into the nature of molecular events and the forces operative within the systems. Therefore, it was attempted to arrive at a tentative evaluation of the molecular mechanism governing the binding process of [Leu]-enkephalin to RMP.

The thermodynamics of [Leu]-enkephalin–RMP association is characterized by a negative change in heat ca-

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pacity ($\Delta C_p°'$) and by a decrease of both $\Delta H°'$ and $\Delta S°'$ with increasing temperature (Figure 8), effects typical for reactions dominated by hydrophobic interactions.[46,47] According to estimates of the heat capacity associated with the exposure to bulk water of various nonpolar groups,[48] the observed value of $\Delta C_p°'$ of −202 cal mol^{-1} deg^{-1} may arise both from the burial of three hydrophobic amino acid side chains of the ligand in the ligand–RMP interface and from an improved burial of some lipophilic side chains of RMP that were ab initio located in a hydrocarbon-like microenvironment. This evaluation is in accord with the assumption of three sites of hydrophobic interactions between [Leu]-enkephalin and RMP (Figure 2a).

If hydrophobic bonding were the only factor that stabilizes the ligand–RMP complex, however, one should expect that ligand binding would give positive $\Delta H°'$ terms within the experimental temperature range because of the endothermicity of the transfer of nonpolar amino acid side chains from water to the hydrophobic interior of a protein at temperatures below 40 to 50 °C.[49-51] The present study, however, shows that the binding to RMP of [Leu]-enkephalin is exothermic ($\Delta H°' < 0$) for temperatures within the range 5–35 °C. Thus, in addition to the above entropy term, some source of enthalpy stabilization must be involved in the process of drug–RMP association. This may arise from weak intramolecular forces, such as hydrogen bonds and van der Waals' contacts, the formation of which proceeds with a favorable enthalpy change. Based on estimates of the energetics of hydrogen bonds and van der Waals' interactions[49,52] the observed enthalpy change of −4.91 kcal mol^{-1} (25 °C) can be tentatively attributed to the formation of two or three H bonds plus several van der Waals' contacts in the low dielectric interior of the ligand–RMP complex. The above interpretations are in line with the principle of protein–protein association and recognition suggested by Clothia and Janin,[53] whereby hydrophobic forces predominantly stabilize protein–protein complexes. On the other hand, H-bonds and van der Waals' contacts represent minor stability factors but they decide which proteins recognize each other.

The pH dependence of [Leu]-enkephalin binding to RMP suggests that ionizing groups with pK_as in the pH range of 4.5–5.0 and of 7.0–7.5 play an important role in ligand–receptor interactions. The former titratable groups may be identified with the side-chain carboxylates of amino dicarboxylic acids of RMP (pK_a = 4.5–4.7; 20 °C, this work). The latter ionizing group may be attributed to the N-terminal amino group of [Leu]-enkephalin (pK_a = 7.4; 20 °C, this work). The pH-dependent binding properties may be interpreted as follows: the protonation of the glutamic acid γ-carboxylates denatures RMP and simultaneously cancels the anionic binding site (Glu-40). To be operative, the above anionic binding site requires a cationic counterpart on the ligand. However, with increasing pH the positively charged amino group of [Leu]-enkephalin is progressively deprotonated, and the affinity for the receptor of the ligand concurrently decreases.

The red-shifted UV difference spectrum caused by binding of [Leu]-enkephalin to RMP is characterized by positive peaks above 270 nm and by a sequence of positive and negative bands between 250 and 270 nm. The observed shifts to longer wavelengths are consistent with the transfer of aromatic chromophores from a polar to a nonpolar environment.[54] The most straightforward interpretation of the spectral changes therefore suggests that some side chains of aromatic amino acid residues initially exposed to bulk water became buried in regions of lower polarity upon formation of the ligand–RMP complex. Tryptophan residues are lacking in both ligand and RMP; therefore, the peaks at 278 and 284 nm can be assigned to a perturbation of the phenolic chromophore in tyrosine, and the "wiggle line" between 250 and 270 nm can be attributed to changes in the microenvironment of the phenyl chromophore in phenylalanine residues. The transfer of a phenolic chromophore from the external environment to the hydrocarbon core of a protein can be related to a difference in molar absorption ($\Delta\epsilon_{287}$) of about 1000 M^{-1} cm^{-1}.[55] Therefore, the burial of the phenolic group of [Leu]-enkephalin in the interior of the ligand–RMP complex may account for most of the observed $\Delta\epsilon_{284}$ value of about 1510 M^{-1} cm^{-1} (20 °C). The remaining 500 M^{-1} cm^{-1} of $\Delta\epsilon_{284}$ may reflect the partial ionization of the phenolic OH group ensuing from the net formation of a hydrogen bond between the tyrosyl side chain of the ligand and a carboxylate function of RMP in a low dielectric hydrophobic pocket.

Binding Properties of Opiate Peptides Other than [Leu]-enkephalin. The free energy change ($\Delta G°'_{278}$) of dynorphin$_{1-8}$ binding to RMP, as calculated from the observed dissociation constant (K_i), was −6.17 kcal mol^{-1}, whereas a $\Delta G°'_{278}$ value of −5.40 kcal mol^{-1} was observed for the formation of the [Leu]-enkephalin–RMP complex. The enhanced stability relative to [Leu]-enkephalin of the dynorphin–RMP system suggests a further binding site of RMP for dynorphin$_{1-8}$. Dynorphin$_{1-8}$ represents a C-terminally extended [Leu]-enkephalin.[37,38] The additional tripeptide Arg-Arg-Ile may give rise to further noncovalent interactions with RMP. Several possible modes of binding are illustrated in Figure 2c. An almost converse state of affairs emerges when the affinity of des-Gly$_3$-[Leu]-enkephalin to RMP is considered. In this case, the free energy change ($\Delta G°'_{278}$) was found to −4.85 kcal mol^{-1}. The increment of $\Delta G°'_{278}$ by 0.55 kcal mol^{-1} relative to [Leu]-enkephalin may be explained by our finding, as deduced from space-filling models, that the truncated [Leu]-enkephalin could not optimally interact with RMP (see above). The binding to RMP of the D enantiomer of the natural [Leu]-enkephalin was accompanied by a free energy change of −4.33 kcal mol^{-1}. Although this observation is indicative for the stereoselective binding to RMP, we had expected a lower affinity of RMP to the *all*-D derivative from model building (see above). The binding strength of *all*-D-[Leu]-enkephalin suggests that more than the anticipated binding facilities were exploited. Possibly, there exist some van der Waals' interactions, the exact nature of which is scarcely predictable. The observed free energy change ($\Delta G°'_{278}$ = −2.70 kcal mol^{-1}) for the binding of naloxone to RMP can be related to two to three noncovalent interactions between ligand and receptor. As can be inferred

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from the proposed binding pattern of PEO (Figure 2b), naloxone cannot profit by the presence of the above-mentioned second and third lipophilic binding site of RMP. The absence of two hydrophobic interactions relative to [Leu]-enkephalin can account for the difference in the free energy change observed with naloxone in comparison to [Leu]-enkephalin. The free energy change ($\Delta G^{\circ\prime}_{278}$) for binding to RMP of three analogues of [Leu]-enkephalin in which the terminal tyrosine moiety was replaced by phenylalanine, phloretic acid (des-NH_2-Tyr), and hydrocinnamic acid (des-NH_2-Phe) was found to be −5.13, −4.71, and −4.10 kcal mol^{-1}, respectively. Hydrogen bonds are commonly considered as contributing little to the stability of ordered structures.[49,52] Therefore, the difference in the free energy change between [Leu]-enkephalin and [Phe_1,Leu]-enkephalin may be attributed to the inability of the latter to interact with RMP via a tyrosine–carboxylate H bond. According to Perutz, the bond energy per salt bridge may be estimated as 1–2 kcal mol^{-1}.[56] Therefore, the reduced binding capacity relative to [Leu]-enkephalin of des-NH_2-[Leu]-enkephalin can probably be ascribed to the absence of the salt bridge between the α-amino group of [Leu]-enkephalin and RMP's anionic binding site in the des-NH_2-[Leu]-enkephalin–RMP complex. The cholecystokinin subunit (CCK-8) can be considered an N- and C-terminally extended variant of [Met]-enkephalin which is designated by the "semitrivial" name Asp-[$Tyr_1(O_\eta SO_3H)$,Met_2,Trp_4,Met]-enkephalyl-Asp-Phe-NH_2. According to Beddell et al.[29] and Morley,[30] each of these "modifications" should lead to a more or less pronounced drop in opiate activity. Therefore, the lack of affinity to RMP observed for CCK-8 is not surprising. In addition to CCK-8, four MSH-related tetrapeptides were used as probes to evaluate the binding specificity of RMP. Only the presumptive "active core" of MSH, H-His-Phe-Arg-Trp-OH, which may bear some structural resemblance with opiate-like peptides, displayed a measurable affinity to RMP ($\Delta G^{\circ\prime}_{278}$ = −2.84 kcal mol^{-1}). These findings demonstrate that RMP does not randomly associate with each and every peptide.

It is well known that opiate peptides bind nonspecifically to many macromolecular systems. Therefore, some control experiments were performed in which RMP was replaced by unrelated peptides of comparable size. The binding specificity of enkephalin-like peptides to RMP could be confirmed by the finding that [Leu]-enkephalin did bind neither to glucagon nor to a synthetic proinsulin analogue consisting of 43 amino acid residues.[57]

Conclusions

The term "receptor", as generally understood in pharmacology, describes a macromolecular compound that is capable both of recognizing and binding a drug and of inducing a biological response. We have designed an opioid receptor mimetic peptide that is able to recognize and bind opiate-like drugs. The binding is saturable, stereospecific, and discriminative for closely related opioid peptides. The low affinity of RMP for naloxone, and presumably for other morphine analogues, may be best rationalized by the preferential use of peptidic ligands as steric probes to design the spatial arrangement of RMP's binding region. The molecular details of the [Leu]-enkephalin–RMP association as tentatively evaluated by equilibrium studies are compatible with the initially designed binding scheme (Figure 2a). RMP's mode of action may provide a working hypothesis of the molecular mechanism governing the binding process of opiate peptides to natural opioid receptors. The affinity of opioid ligands for RMP is several orders of magnitude lower than that for naturally occurring opiate receptors. This finding is not surprising in view of the molecular size of RMP, which merely equals 1% of that reported for natural receptors. Due to their macromolecular nature, biological receptors most likely display a high degree of conformational rigidity that is maintained by a multitude of weak intramolecular interactions. A precisely tailored binding area of strongly restricted mobility is capable of effectively fitting ligands. The conformational equilibrium of the natural receptor in solution is predominantly shifted to a particular type of conformer closely resembling the active conformation. Conversely, a solution of the small-sized, and therefore more flexibly structured, RMP most probably represents an equilibrium mixture of rapidly interconverting conformers, only a limited number of which is present in the "active" conformation. As a result, the effective concentration of active RMP conformers is, by far, lower than the total concentration that comprises all conceivable conformations.

Experimental Section

Materials. All chemicals and solvents were reagent grade. N^{α}-Boc-protected amino acids were purchased from Bachem (Switzerland). Prior to use they were checked for homogeneity by thin-layer chromatography. Naloxone hydrochloride hydrate was obtained from DuPont (Germany). Prepacked LiChrosorb RP-18 (250 × 4 mm) (10 μm) and precoated silica gel plates for high-performance thin-layer chromatography (HPTLC) were puchased from Merck (Germany). The following solvents were used in HPTLC: A, chloroform/methanol (3:1); B, chloroform/methanol/acetic acid (45:4:1). Trypsin (EC 3.4.31.4), α-chymotrypsin (EC 3.4.21.1), prolidase (EC 3.4.13.9), and leucine aminopeptidase (EC 3.4.11.1) were obtained from Sigma Chemical Co. (Germany). Carboxypeptidase Y (EC 3.4.16.1) was purchased from Boehringer (Germany).

General Methods. Acid hydrolyses were performed in 6 N HCl in evacuated, sealed tubes at 110 °C for 24 h. Peptide-resins were hydrolyzed according to Gutte and Merrifield.[58] CD measurements were carried out under constant nitrogen flush at 21 °C in an Autodichrograph Mark V (Jobin Yvon, France), fitted with attachments for temperature-controlled runs. The CD data were expressed in terms of mean residue ellipticity (θ). The mean residue weight (MRW) of the artificial receptor was determined from its amino acid composition. Calculation of the CD spectra was based on standard spectral data[45] for the secondary structural elements, of which the percentage contribution to the secondary structure of RMP was determined by predictive methods.[22] On the basis of the reference CD spectra of the four main-chain conformations,[45] the contributions to the observed CD spectra of the various secondary structural elements were determined by a linear least-squares estimate.

Special Syntheses. The protease-catalyzed synthesis of [Leu]- and [Met]-enkephalin, des-Gly_3-[Leu]-enkephalin, $dynorphin_{1-8}$, the cholecystokinin subunit (CCK-8), and the MSH fragments, and the preparation of an *all*-D-[Leu]-enkephalin has been described elsewhere.[40,59-63] The enzymatic synthesis of [Phe_1,Leu]-enkephalin (this work) corresponded to the procedure described for the preparation of [Leu]-enkephalin.[59] Des-NH_2-[Leu]-enkephalin and des-NH_2-[Phe_1,Leu]-enkephalin were synthesized starting from the enzymatically prepared tripeptide H-Gly-Phe-Leu-N_2H_2Ph[59] by a stepwise procedure using DCC and HOBt as condensing agents. The coupling of Boc-Gly-OH and the subsequent deacylation step were followed by acylation of the resulting tetrapeptide with 3-(4-hydroxyphenyl)propionic acid

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and 3-phenylpropionic acid, respectively. Removal of the protecting groups was accomplished as described previously[59] (1-^{14}C)-Glycine (0.05 mCi/mmol) was obtained from Amersham (Germany) and was N^{α}-acylated as described by Moroder et al.[64] to give N^{α}-Boc-(1-^{14}C)-Gly-OH. (*3-Gly*-1-^{14}C)-[Leu]-enkephalin (7×10^9 cpm/mol) was prepared according to method A, as reported elsewhere.[59] N^{α}-Boc-N^{δ}-phthaloylornithine [Boc-Orn-(Pht)-OH] was prepared analogously to the synthesis of Boc-Lys(Pht)-OH[65] via catalytic dehydrogenation of Boc-Orn(Z)-OH to remove the benzyloxycarbonyl group and subsequent phthaloylation of the δ-amino group with *N*-carbethoxyphthalimide:[66] yield 74%; HPTLC R_f (A) 0.63, R_f (B) 0.86; $[\alpha]^{20}_D$ +8.4° (*c* 1.5, MeOH); mp 142–145 °C. Anal. ($C_{18}H_{22}N_2O_6$) C, H, N. Boc-Asp(OBzl)-Pro-OH and Boc-Ser(Bzl)-Pro-OH were prepared from Boc-Asp(OBzl)-OH and Boc-Ser(Bzl)-OH, respectively, and H-Pro-OMe according to the one-pot procedure described by König and Geiger.[67] The regiospecific esterase potential of carboxypeptidase Y (CPD-Y) was exploited to selectively remove the methyl ester of the proline residues. The dipeptides were incubated in the presence of CPD-Y for 5 h [enzyme/substrate ratio 1:1000 (M/M)]. The reaction conditions followed those described by Widmer and Johansen for CPD-Y-catalyzed peptide synthesis.[68] Boc-Asp(OBzl)-Pro-OH: yield 60%; HPTLC R_f (A) 0.84; R_f (B) 0.60; $[\alpha]^{20}_D$ −30.5° (*c* 1, MeOH); mp 99–101 °C. Anal. ($C_{21}H_{28}N_2O_7$) C, H, N. Boc-Ser(Bzl)-Pro-OH: yield 63%; HPTLC R_f (A) 0.80, R_f (B) 0.79; $[\alpha]^{20}_D$ −44.8° (*c* 1.1, MeOH). Anal. ($C_{20}H_{28}N_2O_6$) C, H, N.

Synthesis of Protected [Orn$_{1,7,24,30}$]RMP. In order to obviate the difficulties frequently encountered during the synthesis of arginyl peptides,[69] N^{δ}-phthaloylated ornithine residues were incorporated into the growing peptide chain as temporary substitutes for arginine residues. The synthesis of Boc-Orn(Pht)-Thr-(Bzl)-Ile-Thr(Bzl)-Val-Thr(Bzl)-Orn(Pht)-Ser(Bzl)-Pro-Gly-Glu-(OBzl)-Thr(Bzl)-Phe-Thr(Bzl)-Phe-Thr(Bzl)-Glu(OBzl)-Ser-(Bzl)-Pro-Thr(Bzl)-Asp(OBzl)-Pro-Gly-Orn(Pht)-Thr(Bzl)-Phe-Thr(Bzl)-Phe-Thr(Bzl)-Orn(Pht)-Ser(Bzl)-Pro-Gly-Glu(OBzl)-Thr(Bzl)-Phe-Thr(Bzl)-Val-Thr(Bzl)-Glu(OBzl)-resin followed the stepwise solid-phase procedure.[70] Attachment of Boc-Glu-(OBzl)-OH as its tetramethylammonium salt (2.0 g) to a chloromethylated styrene–divinylbenzene resin (2.5 g) (Bio-Beads S-X1) was accomplished as described previously:[71,72] yield 0.34 mmol of glutamic acid per gram of resin. Starting from Boc-Glu(OBzl)-resin (1.12 g, 0.34 mmol), the assembly of the peptide chain proceeded according to the following protocol: (a) 50% TFA/CH_2Cl_2, 1 min; (b) repeat step a, 20 min; (c) CH_2Cl_2, 2 min, six times; (d) *i*-PrOH, 2 min, twice; (e) repeat step c; (f) 5% 4-methylmorpholine/CH_2Cl_2, 1 min; (g) repeat step f, 6 min; (h) repeat step c; (i) 1% *N,N*-diisopropylethylamine/CH_2Cl_2; (j) repeat step c; (k) repeat step d; (l) repeat step c; (m) 3 equiv of Boc-protected amino acid/CH_2Cl_2 for 10 min, then 3 equiv of DCC/CH_2Cl_2, 2 h; (n) repeat steps c–e; (o) repeat steps f–n, except step n, which is changed to 2 equiv of each Boc-protected amino acid and DCC, 1 h; (p) 1 equiv of *N,N*-diisopropylethylamine for 2 min, then 4 equiv of acetic anhydride/CH_2Cl_2, 15 min; (q) repeat step c, three times; (r) repeat step d; (s) repeat step c.

In order to prevent a possible N^{α}-alkylation by excess resin-bound chloromethyl groups of proline residues,[73] the latter were introduced as integral constituents of dipeptides. Boc-Asp-(OBzl)-Pro-OH and Boc-Ser(Bzl)-Pro-OH were coupled as the hydroxybenzotriazole esters.[67] The protected dipeptides (4 equiv) were reacted in DMF in the presence of DCC and HOBt (4 equiv each) at 0 °C for 30 min, and the resulting precipitate was filtered off. The activated dipeptides were then added to the peptide resin (4 h), and the above schedule (including a second coupling step) was resumed.

Preparation of [Orn$_{1,7,24,30}$]RMP. The completed peptide resin was dried in vacuo over P_2O_5 to give 2.49 g (220 μmol) of the protected peptide in 65% yield. The peptide resin was dissolved in TFA and treated with HBr for 1 h in order to cleave the peptide from the resin and to remove the protecting groups, except the phthaloyl groups of ornithine. After removal of the solvents, the residue was dissolved in 0.05 M NH_4HCO_3 and adjusted to pH 8.0 with NH_4OH. After lypophilization, the crude tetracontapeptide was desalted on a Bio-Gel P-2 column and freeze-dried: yield 846 mg of [Orn(Pht)$_{1,7,24,30}$]RMP (176 μmol, 80%).

In order to remove the N^{δ}-phthaloyl group from the ornithine residues, the lyophilized material was added to a 4 M solution of hydrazine acetate in methanol (25 mL). After 25 h at 40 °C, the solution was diluted with water (50 mL) and adjusted to pH 3.5 with AcOH. The insoluble (4 °C) phthalhydrazide was removed, and the solution was lyophilized. The product was subjected to gel filtration on a Bio-Gel P-6 column in 0.05 M NH_4HCO_3. The material represented by one major peak was isolated and concentrated as described above. Further purification was achieved by chromatography on the anion exchanger AG 1-X2 (acetate cycle) (Bio-Rad). The column was eluted with a linear gradient from MeOH/0.05 M AcOH (1:9) to MeOH/0.25 M AcOH (1:9). Ninhydrin test and thin-layer chromatography served to locate the desired material in 0.15 M acetic acid eluates. Two minor but substantial peaks followed on elution with 0.2 M acetic acid. According to amino acid analyses, they represented by-products originated from incomplete dephthaloylation. The desired fractions were pooled and lyophilized to give 386 mg of [Orn$_{1,7,24,30}$]RMP (90 μmol, 51%). The dephthaloylated product, although not completely homogeneous, was then subjected to guanidination in order to convert the ornithine residues to the respective arginine residues.

Preparation of RMP via Guanidination.[74,75] [Orn$_{1,7,24,30}$]RMP (386 mg, 90 μmol) was dissolved in 10 mL of water and then added to a solution of 1.5 M *O*-methylisourea in 10 mL of water that had been adjusted to pH 11 with NaOH. After 8 days the solution was acidified to pH 5.0 with AcOH and desalted on a Bio-Gel P-2 column with 0.05 M NH_4HCO_3 as eluant. The material represented by a major peak was isolated by lyophilization and subsequently subjected to partition chromatography on silica gel 60 as described previously.[76] The elution profile showed a minor peak with R_f 0.27 and a major peak with R_f 0.22. The minor product (~10%) was ninhydrin positive and Sakaguchi negative—obviously, the ornithine residues had not been converted to arginine residues. The major product (~90%), which was ninhydrin and Sakaguchi positive, was lyophilized and further purified via ion-exchange chromatography on AG 1-X2 as described above. The Sakaguchi test, thin-layer chromatography, and amino acid analysis served to locate the desired product in 0.11 M acetic acid. Additional Sakaguchi-positive material (~30% of the target product) was eluted with 0.12–0.14 M acetic acid. According to amino acid analyses, those byproducts contained ornithine as well as arginine residues. The fractions containing the target product were pooled and lyophilized. This material was further chromatographed via reversed-phase HPLC on a LiChrosorb RP-18 column. Elution was performed with a linear gradient of acetonitrile (0.75%/min), starting with 0.1 M $NaClO_4$/0.005 M phosphate buffer, pH 7.4 (flow rate 1 mL/min). A major peak (t_R 76 min) and three substantially smaller peaks (t_R 62, 71, and 79 min, respectively) were detected. Amino acid analyses showed that the minor peaks represented byproducts. Rechromatography of the desired product on the LiChrosorb RP-18 column gave a single, symmetrical peak (t_R 77 min; capacity

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factor (K') = 37.5]. After desalination on a Bio-Gel P-2 column (see above) and lyophilization, 134 mg of RPM was obtained (30 μmol, 33%): overall yield (based on the crude peptide-resin) 13.6%. The synthetic tetracontapeptide was homogeneous with regard to thin-layer chromatography and electrophoresis: HPTLC using the solvent systems 1-butanol/pyridine/acetic acid/water (20:10:3:10) and ethyl acetate/pyridine/acetic acid/water (5:5:1:3) each gave a single spot (ninhydrin and Sakaguchi detection) with R_f values of 0.25 and 0.34, respectively; electrophoreses in pyridinium acetate buffer (pH 3.45 and 6.50, 400 V, 30 min) on precoated cellulose plates each gave a single spot (ninhydrin and Sakaguchi) with R_f values of 0.42 and −0.13 relative to arginine. Amino acid analysis of an acid hydrolysate gave the following: Asp, 1.04 (1); Thr, 12.25 (13); Ser, 2.75 (3); Glu, 4.12 (4); Pro, 4.08 (4); Gly, 3.00 (3); Val, 1.97 (2); Ile, 0.94 (1); Phe, 5.18 (5); Arg, 3.90 (4). Complete enzymic digestion successively with trypsin, α-chymotrypsin, prolidase, and then with leucine aminopeptidase gave the following: Asp, 1.02 (1); Thr, 12.58 (13); Ser, 3.02 (3); Glu, 4.08 (4); Pro, 3.90 (4); Gly, 3.00 (3); Val, 1.96 (2); Ile, 0.97 (1); Phe, 5.07 (5); Arg, 3.96 (4).

Measurements of Ligand Binding. Equilibrium Dialysis. Dialysis studies were performed in cylindrical Teflon cells having a total capacity of 11.5 mL and which were divided into two equal compartments by a semipermeable membrane of 1.35-cm radius. The membranes were cut from cellulose dialysis tubings (Spectrapor 3, Spectrum Medical Industries Inc.). The permeability of the membrane to the synthetic receptor was found to be ~4% within the experimental time (48 h). The binding data were corrected for the leakage effect. Retention of the ligands to the membrane was found to be negligible. The experiments were generally started by placing 5 mL of an RMP solution in 10^{-2} M sodium phosphate buffer (pH 7.0) to one side of each cell and 5 mL of an equally buffered solution of the ^{14}C-labeled ligand to the other side of the cell. The dialysis cells prepared in this manner were shaken with a frequency of 25 oscillations per minute. After equilibration, aliquots (5 μL) were taken from each side of the cells and the concentration of the ^{14}C-labeled ligand was determined on a Packard Tri-Carb 3380 counter. Each dialysis experiment was run in duplicate. Amino acid analyses served to determine the peptide concentration. The amounts of bound and free ligands were calculated as described by Changeux et al.[77]

Gel Filtration.[78] In a typical binding experiment, a 0.8 × 60 cm column of Sephadex G-15, thoroughly equilibrated with a known solution of [Leu]-enkephalin in 10^{-2} M sodium phosphate buffer (pH 7.0), was loaded with a known solution of RMP in 1 mL of the equilibrating buffer. The elution rate was 10 mL/h, and the column effluent was monitored spectrophotometrically at 280 nm. The elution pattern displayed a first peak representing the free receptor and the ligand–RMP complex, followed by a trough (negative peak) whose area provided a measure of the amount of ligand bound to the receptor.[78]

UV Difference Spectral Measurements. UV absorptiometry at constant temperature was performed in a Cary 118 double-beam spectrophotometer, equipped with thermostatted reference and sample compartments. In a typical experiment, the peptide solutions were placed in two-celled cuvettes. On the reference side, one side of the cuvette held a solution of [Leu]-enkephalin in a 10^{-2} M sodium phosphate buffer, and the other side held an equally buffered solution of RMP. On the sample side, one side held [Leu]-enkephalin and RMP mixed at the same concentrations as in the reference cuvette, and the other held buffer. The mixture was allowed to react to equilibrium in the spectrophotometer, and the difference spectrum was recorded. Equilibrium constants were derived from the variations at distinct wavelengths of the difference spectra with varying ligand concentrations according to a method described by Rosotti and Rosotti.[42]

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Registry No. RMP, 87937-17-5; [Leu]-enkephalin, 58822-25-6; [Met]-enkephalin, 58569-55-4; des-Gly_3-[Leu]-enkephalin, 60254-81-1; $dynorphin_{1-8}$, 75790-53-3; *all*-D-[Leu]-enkephalin, 87921-92-4; [Phe_1,Leu]-enkephalin, 60254-86-6; des-NH_2-[Leu]-enkephalin, 87902-07-6; des-NH_2-[Phe_1,Leu]-enkephalin, 87902-08-7; N^{α}-Boc-(1-^{14}C)-Gly-OH, 66025-27-2; (3-*Gly*-1-^{14}C)-[Leu]-enkephalin, 87902-09-8; H-His-Phe-Arg-Trp-OH, 4289-02-5; Boc-Orn(Pht)-OH, 87902-10-1; Boc-Orn(Z)-OH, 2480-93-5; Boc-Asp(OBzl)-Pro-OH, 68939-29-7; Boc-Ser(Bzl)-Pro-OH, 87902-11-2; Boc-Asp(OBzl)-OH, 7536-58-5; Boc-Ser(Bzl)-OH, 23680-31-1; H-Pro-OMe, 2577-48-2; Boc-Asp(OBzl)-Pro-OMe, 87902-12-3; Boc-Ser(Bzl)-Pro-OMe, 76947-97-2; Boc-Glu(OBzl)-O^-·Me_4N^+, 87902-14-5; [$Orn_{1,7,24,30}$]RMP, 87937-16-4.

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Replacement of the Peptide-Backbone Amides Connecting Tyr-Gly and Gly-Gly in Leucine-enkephalin with Ketomethylene Groups: Synthesis and Biological Activity

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A peptide analogue of Leu-enkephalin was synthesized in which the amide linkages between Tyr-Gly and Gly-Gly were replaced by ketomethylene groups. The resulting analogue, **12**, had $^1/_{4000}$th and $^1/_{2400}$th the opiate receptor binding activity of Leu-enkephalin when (^3H)[D-Ala^2,D-Leu^5]enkephalin and (^3H)naloxone, respectively, were used as tritiated ligands. When tested for analgesia in mice by the tail-flick assay, **12** produced analgesia in 50% of the mice tested at a dose of 24.3 μg/mouse (icv), while the ED_{50} of Leu-enkephalin is 240 μg/mouse (icv). At a dose of 40 μg/mouse (icv) or higher, **12** caused convulsions in a dose-dependent manner. No analgesia was observed after intravenous (iv) administration of 240 μg/mouse of **12**.

Several theories have been advanced concerning the contribution of the peptide-backbone amide groups in enkephalins to proper binding to the analgesic receptor. In 1980, Hudson et al.[1] proposed a conformational model for the binding of Met-enkephalin to the analgesic receptor site. This model was supported by the analgesic receptor binding activity of some Met-enkephalin analogues that they had synthesized. In this model the peptide backbone was involved in receptor binding at only two points. These binding points were the terminal amine group and the carbonyl group that is part of the amide linkage connecting the two glycines.

Additional support for the Hudson et al.[1] model has been provided by studies with enkephalin analogues with trans olefin groups in place of peptide-backbone linkages.

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