Articles

Mapping the Active Site of Angiotensin-Converting Enzyme by Transferred NOE Spectroscopy

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The interaction of five furylacryloyl (fa)-amino acid derivatives, fa-Phe, fa-Phe-Phe, fa-Gly-Leu-NH_2, fa-Ala-Lys, and fa-Trp, with angiotensin-converting enzyme (ACE), a protein of MW = 130 kDa, was studied by transferred NOESY experiments. Identification of fa derivatives binding to ACE as well as determination of their relative affinities could be accomplished directly from the compound mixtures. Of the five fa derivatives we found that fa-Phe, fa-Trp, and fa-Gly-Leu-NH_2 bind more strongly to ACE than the other two. The dissociation constant of fa-Phe was determined from NMR spectra to 5×10^{-4} M. A large excess of dipeptides competitively displaced fa-Trp and fa-Phe-Phe from the receptor pocket, allowing the binding site to be mapped. Also, the relative affinities of the fa-Phe, fa-Ala-Lys, and fa-Gly-Leu-NH_2 changed after addition of the dipeptides with fa-Gly-Leu-NH_2 showing the strongest binding. In addition, the presence of a strong inhibitor of the S1′ and S2′ sites, namely captopril, resulted in the same transferred NOE intensities of fa-Phe, indicating that it binds solely to the S1 and S2 subsites. A rapid screening of binding specificity from mixtures is possible by using a large excess of ligand(s) in transferred NOE studies, even when relatively small amounts of protein are present.

Introduction

Angiotensin-converting enzyme (ACE; EC 3.4.15.1) is a dipeptidyl carboxypeptidase of relatively broad specificity.[1] It is a metalloprotease requiring a zinc atom for catalyzing the conversion of the inactive decapeptide angiotensin I to the potent vasopressor octapeptide angiotensin II by cleavage of the carboxy-terminal dipeptide.[2] In addition, it inactivates the vasodepressor nonapeptide bradykinin and thus plays a central role in blood pressure regulation of man.[3,4] Several potent orally active inhibitors with nanomolar inhibition constants are effective in the treatment of hypertension and congestive heart failure.[5,6] Captopril, a mercaptoalkanoyl amino acid, was the first ACE inhibitor used in hypertension treatment.[7,8]

The search for inhibitors with novel properties has proven to be time-consuming due to the fact that no crystal structure of the enzyme is available. A rational design of inhibitors based on exact binding site characteristics has been difficult to date. As drug discovery often relies on a binding site model inferred from a number of different ligands binding to the receptor pocket, interpretation of the transferred nuclear Overhauser effect (trNOE) data provides an alternative solution.[9,10] A new approach to map the active site of ACE by "scanning" the active site with several weakly binding ligands by trNOE methods is introduced. We used the binding site model by Cushman and Ondetti, who proposed that the ACE binding site comprises of the catalytically active zinc atom, at least two subsites on the amino terminal end to the left, and two subsites on the carboxy terminal end to the right of the zinc atom as the basis for our hypothesis (cf. Figure 1).[7] Additional subsites may be defined with regard to the amino-terminal end of the receptor pocket. Essential for a good ACE inhibitor is effective binding to the S2′ subsite at the carboxy-terminal end of the receptor pocket and strong interactions with the zinc atom. For the S2′ subsite, proline was shown to be the most effective and almost all commercially existing inhibitors bear this residue.[11,12]

We have previously reported that trNOE measurements can be efficiently used to identify ligands from a mixture of components having binding affinity for a protein.[13,14] Small molecules (MW < 1500 Da) in the free state yield small, positive NOEs, while large molecules, e.g. proteins (MW > 10 kDa), yield large, negative NOEs. Ligands reversibly binding to a biomacromolecule are labeled with information of the large protein and, thus, give rise to large and negative NOEs, which reach their maximum at short mixing times (Figure 1). Therefore, binding of ligands to a large biomolecule can be identified by the sign, size, and buildup rate of these so-called transferred NOEs. Because of the need for an averaging effect, brought about by fast chemical exchange on the NMR time scale, trNOE experiments are limited to ligands with dissociation constants (K_D) in the range between 10^{-3} and 10^{-7} M. The spectral properties of excess ligands in solution are evoked by small fractions of bound molecules, greatly enhancing

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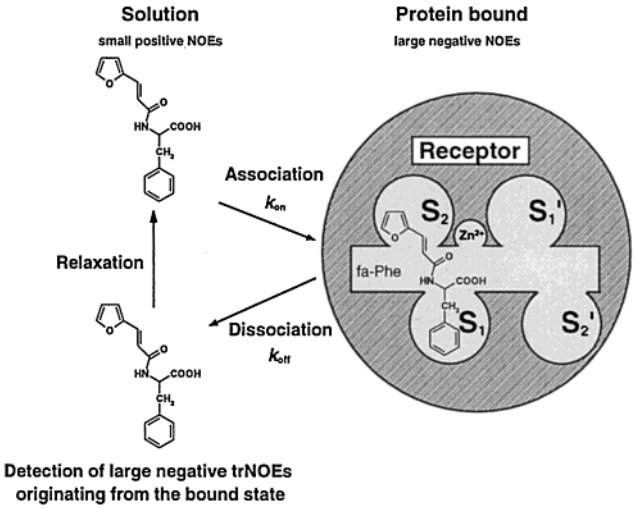


Figure 1. Upon dissociation of the ligand from the protein, NOE information concerning the 3D structure of the ligand in the bound state is transferred into solution. This trNOE effect can be used to determine the 3D-structure of the ligand in the bound state, as well as the fact that binding occurred and, under certain conditions, even the binding strength. In the study conducted here no change of conformation occurred from the bound to the free state. However, trNOE intensities could be used to determine K_D values. The schematic arrangements of the proposed ACE binding site[8] with fa-Phe binding to the S1 and S2 subsites is shown.

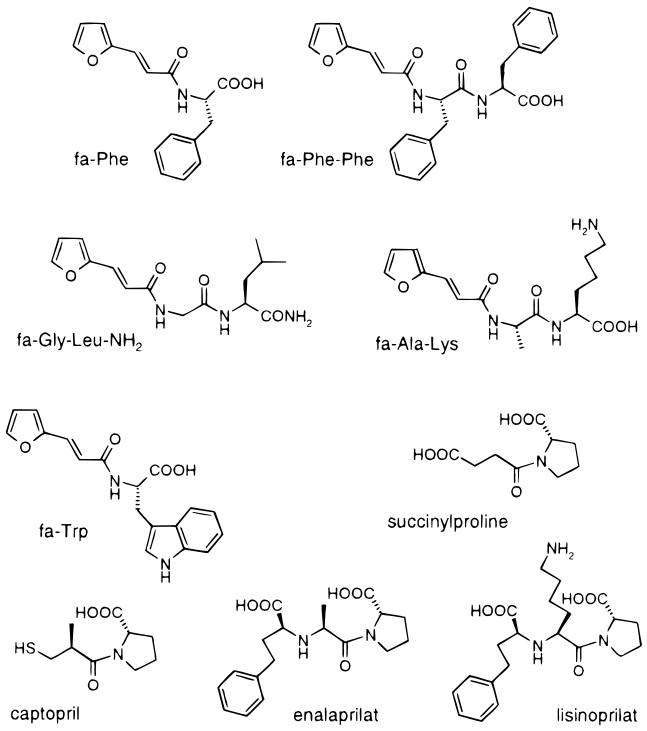
sensitivity. An additional advantage arises from the fact that identification of the chemical structure and the conformation of the ligand in its bound state is directly possible from the NMR experiment.[15]

Results and Discussion

To gather information about the S1, S2, S1′, and S2′ subsites of the active site we used the product of the ACE-catalyzed hydrolysis of fa-Phe-Gly-Gly (fa = furylacryloyl), namely fa-Phe (Chart 1), as the starting point for our study. Fa-Phe-Gly-Gly presumably binds with the fa group to the S2 subsite, the Phe residue to the S1 subsite (Figure 1), whereas the carboxy-terminal dipeptide Gly-Gly binds to the S1′ and S2′ subsites.[16,17]

Analysis of Transferred NOE Data. The NOESY spectrum in Figure 2 recorded after addition of fa-Phe-Gly-Gly to a sample containing angiotensin-converting enzyme (ACE) reveals that the substrate is entirely hydrolyzed and that only fa-Phe gives rise to trNOEs, as indicated by the negative sign (blue signals) of the ligand's intramolecular NOEs. A reference spectrum recorded in the absence of ACE revealed that the unhydrolyzed substrate fa-Phe-Gly-Gly yields cross-peaks of the opposite sign compared to the diagonal, namely positive NOEs. One can also detect a positive NOE (red signal) from the other product of the hydrolysis, the nonbinding Gly-Gly dipeptide present in the sample (Figure 2). Therefore, the addition of ACE to the sample accounts not only for the hydrolysis of the substrate but also for the change of NOE sign of the fa-Phe molecule. Analysis of the NOE buildup curves gives further evidence of the binding process. TrNOEs reach their maximum fast while NOEs of nonbinding molecules have much slower build up rates.[13] In agreement, the NOE of the cross-peak between the NH_{Phe} and the a2 proton of the ligand in the presence of ACE

Chart 1. Structures of the Five fa Derivatives, i.e., fa-Phe, fa-Phe-Phe, fa-Gly-Leu-NH_2, fa-Ala-Lys, and fa-Trp; Succinylproline; and Three Commercially Available ACE Inhibitors, i.e., Captopril, Enalaprilat, and Lisinoprilat



reaches its maximum at mixing times of about 110 ms (Figure 3a), in comparison to 700 ms for the uncomplexed fa-Phe-Gly-Gly (Figure 3b). Also, trNOEs usually have a much larger intensity than the corresponding NOEs of the free ligands. The trNOE of fa-Phe in the presence of ACE reaches its maximum value of ≈−10%, while the same cross-peak reaches only 0.6% when no receptor is present. Thus, the trNOE is about 16 times stronger than the NOE of the free ligand. The analysis of the trNOEs revealed that the conformation of the ligand in the bound state does not deviate from that in the free state. This was expected since the furylacryloyl group has a rigid, planar structure permitting little conformational freedom. NOESY spectra with good signal-to-noise ratios could be recorded within about 1.5 h, since the absolute magnitude for trNOEs is greater than that for normal, positive NOEs.

Determination of the Dissociation Constant by Line-Shape Analysis. Further evidence of the binding process was obtained from 1D ^1H NMR spectra. In the absence of enzyme, the ligands have sharp resonance line shapes characteristic for small molecules, while in the presence of ACE line-broadening effects are caused by T_2-relaxation rate contribution originating from the bound state.[18−20] The magnitude of line broadening depends on the fraction of ligand bound to the protein. Exchange processes did not contribute to the observed line broadening, since the chemical shifts of the signals did not differ on increasing the ligand concentration (Figure 4). This is characteristic for fast exchange behavior, and under these conditions K_D may be obtained by plotting $1/\Delta\nu_{1/2}$ ($\Delta\nu_{1/2}$, difference in line width at half-height of the bound and free state) versus ligand concentration (Figure 5). Titration of fa-Phe resulted in

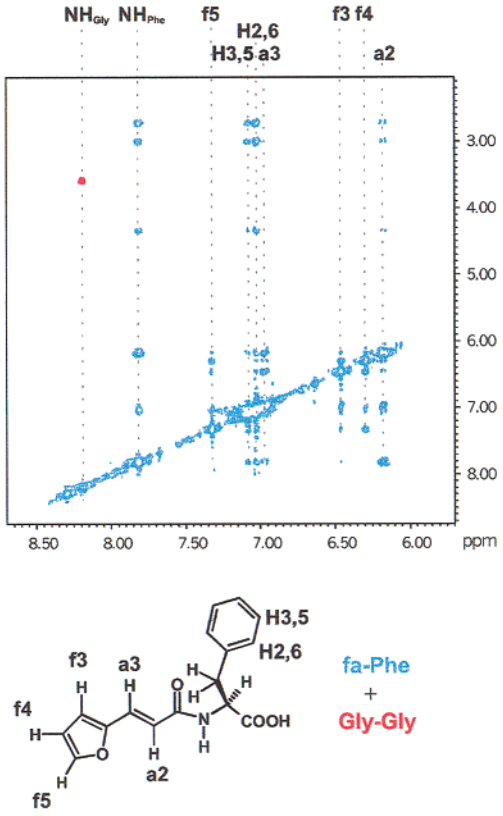


Figure 2. NOESY spectrum of fa-Phe and Gly-Gly in the presence of 40 nmol ACE showing the connectivities from the NH and aromatic protons. All cross-peaks arising from fa-Phe have the same sign as the diagonal (blue signals), indicating binding affinity. The cross-peak of the nonbinding Gly-Gly dipeptide has an opposite sign, revealing the positive NOE (red signal). Identification of the binding molecule from this mixture is therefore possible by observing the sign of the NOE cross-peaks.

an absolute binding constant of $K_D \approx 5 \times 10^{-4}$ M. This value agrees with K_D values from literature.[16]

Analysis of Competitive Binding of Five fa Derivatives. We chose fa derivatives with various carboxy-terminal amino acid residues, which like fa-Phe are not hydrolyzed by ACE, to gather from NOESY spectra further information of the binding specificities of the protein. Four other fa derivatives, fa-Phe-Phe, fa-Gly-Leu-NH$_2$, fa-Ala-Lys, and fa-Trp, in this order, were successively added to the sample, thereby producing four different compound mixtures. All of the added compounds gave rise to trNOEs, thus revealing their binding affinity to ACE. Shown here is the NOESY spectrum of the mixture of all five fa derivatives in the presence of ACE (Figure 6). However, the intensities of the trNOE signals changed when additional fa derivatives were added to the sample and had different magnitudes for the individual compounds. The trNOE cross-peak intensities reflect the relative binding strengths of the derivatives, if certain conditions are fulfilled. As long as all competitive ligands are in the fast exchange regime, the stronger binding inhibitor occupies more binding sites and thus yields larger trNOE cross-peak intensities.[18] Therefore, addition of competitively binding substances leads to changes of the observed trNOE intensities, because the fraction of bound ligands is reduced. Determination of relative binding constants is possible, because the trNOE intensity does not change for k_{off} rates ranging from 10^2 to 10^5 s^{-1} when using short mixing times, as was done here ($\tau_m = 100$ ms).[21−23] Longer mixing times would lead to contributions from spin diffusion and give rise to problems at k_{off} rates in the region of 100−500 s^{-1}.[24,25] To exemplify this, the NOE buildup curve of the cross-peak of the NH$_{Phe}$−a2 proton pair, which have a distance of 2.44 Å from each other is shown in Figure 3a (squares). It is the buildup curve of the proton pair used for referencing the binding affinities and shows a very steep increase even at very short mixing times and reaches a maximum NOE value of ≈−10%. The time course for this signal shows no discernible contribution from spin diffusion which would lead to a sigmoid shape.

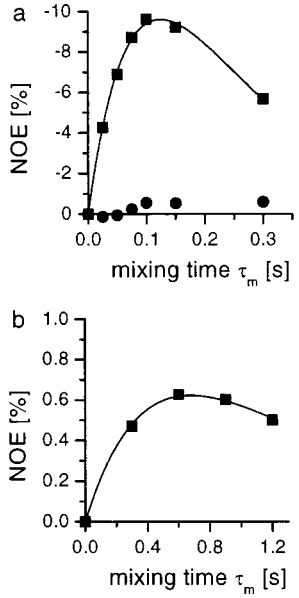


Figure 3. (a) Transferred NOE buildup curves of the cross-peaks corresponding to the NH$_{Phe}$−a2 proton pair (squares) and the a3−f4 proton pair (circles) in the presence of ACE, respectively. The buildup curve for the NH$_{Phe}$−a2 proton pair is indicative of no or little spin diffusion. In contrast, the curve for the a3−f4 cross-peaks shows the typical lag phase common for cross-peaks arising from spin diffusion via a third proton. (b) NOE buildup curve of the substrate fa-Phe-Gly-Gly of the NH$_{Phe}$−a2 proton pair in absence of ACE. No NOE of the a3−f4 proton pair could be detected in the absence of ACE. The fitting function for the NOE buildup curves was $f(t) = p_0 e^{-p_2 t}(1 - e^{-p_1 t})$ and is shown as a solid line. Comparison of the curves reveals the three characteristic differences of normal and transferred NOE buildup curves of low molecular weight substances: opposite sign, larger absolute NOE value, and faster build up of magnetization.

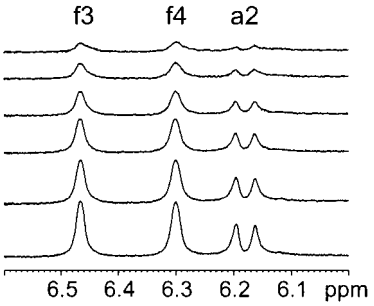


Figure 4. Region of the ^{1}H spectra showing the f3, f4, and a2 signals of fa-Phe in the presence 57 μM ACE. Protein ligand ratio from top to bottom: 1:2, 1:4, 1:8, 1:12, 1:16, and 1:20. No changes in chemical shift upon increase of ligand can be detected.

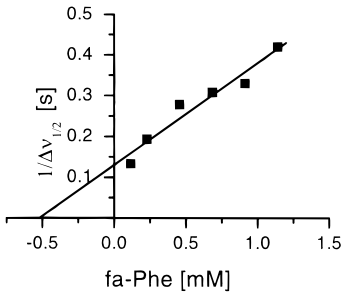


Figure 5. Concentration dependence of the reciprocal broadening of the f3 resonance of fa-Phe. ACE concentration was 57 μM and fa-Phe was added to give a 2-, 4-, 8-, 12-, 16-, and 20-fold ligand excess. From the horizontal linear intercept one can determine K_D.[19,20]

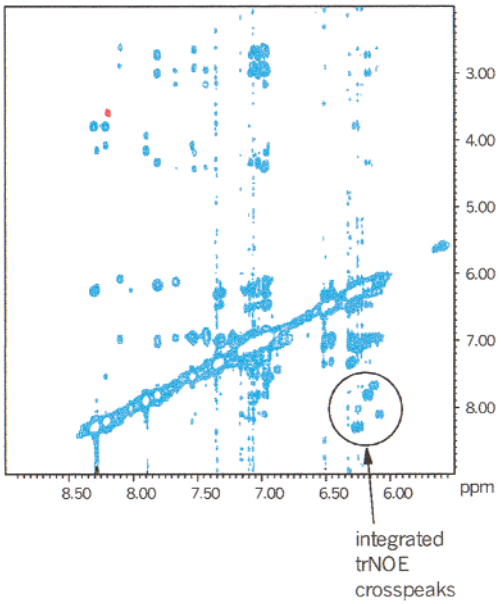


Figure 6. NOESY of a mixture of five ligands (fa-Phe, fa-Gly-Leu-NH_2, fa-Phe-Phe, fa-Trp, and fa-Ala-Lys) in the presence of ACE. The red cross-peak indicates the positive NOE of the nonbinding Gly-Gly dipeptide. All other NOE cross-peaks have a negative sign (blue), revealing their affinity to the ACE receptor. The large circle indicates the region of those cross-peaks used to quantitate relative affinities.

In contrast, the buildup curve of the cross-peak of the a3−f4 proton pair, which is also shown in Figure 3a (circles), has clearly a sigmoid shape. It shows the typical lag phase that can be observed if magnetization is transferred via a relay proton. The distance between the two protons is 5 Å. Here, the magnetization may be relayed by a proton of the protein or the f3 proton situated at half distance between the f4 and a3 protons. These findings indicate that spin diffusion contributes very little or nothing to the cross-peak intensity of the NH_{Phe}−a2 proton pair. Therefore, we have not taken the effect of spin diffusion into account. A rigorous account of spin diffusion was only possible if a fully deuterated protein had been available. However, because there is no expression system for the glycoprotein ACE available as of yet, this is not an option.

From the line-shape analysis performed we know that there was no contribution of chemical exchange to the line width (Figure 4). This is characteristic for fast exchange. Also, the experimentally determined K_D value of 5×10^{-4} M for fa-Phe (which had the highest affinity as shown below) agrees with a fast exchange condition.[25]

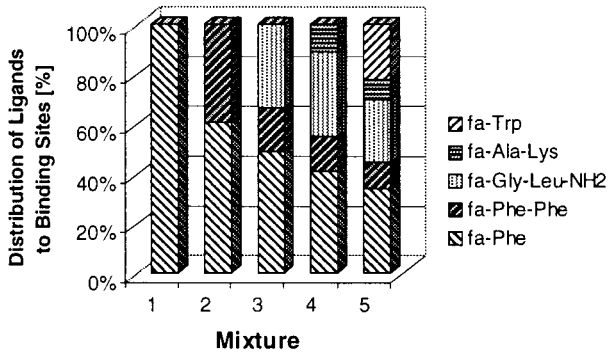


Figure 7. Distribution of fa-Phe, fa-Phe-Phe, fa-Gly-Leu-NH_2, fa-Ala-Lys, and fa-Trp to the available binding sites as obtained from integrating trNOE cross-peaks in five separately recorded NOESY spectra. Mixture 1 contains fa-Phe only; therefore, 100% of the receptor sites are occupied by this ligand. On sequential addition of other ligands (mixtures 2−5), competition for available receptor sites leads to changing distributions for the individual ligands.

Therefore, under the experimental conditions, the trNOE intensity for competitively binding ligands with k_{off} rates ranging from 10^2 to 10^5 s^{-1} is essentially only dependent on the dissociation constant K_D. Potential ligands with $k_{off} > 10^5$ s^{-1} have a very low affinity ($K_D > 10^{-2}$ M) and would produce no trNOEs. On the other hand, high affinity ligands with $k_{off} < 10^2$ s^{-1} lead to a displacement of weaker binding ligands from the active site, which would be easily detected due to a significant reduction of trNOE cross-peaks of lower affinity ligands. Hence, the indirect detection of a high affinity ligand by a marker ligand with known binding characteristics is valuable. In this system, however, none of the fa ligands had an affinity high enough to completely displace the other ligands.

The determination of relative binding affinities of the five fa derivatives was attained by integration of NOESY cross-peaks of the NH amide proton to the a2 proton of the acryloyl moiety (Figure 2). This was the only cross-peak which was well-dispersed and has the same interproton distance in all fa derivatives. Every time a ligand is added, the distribution of the ligands on available binding sites is determined by recording a NOESY spectrum and integrating the indicated cross-peaks (Figure 6). The diagram shows the distribution of the ligands among the binding sites (Figure 7). Relative binding constants were in the order fa-Phe > fa-Trp ≈ fa-Gly-Leu-NH_2 > fa-Ala-Lys ≈ fa-Phe-Phe. Figure 7 illustrates the decrease in fraction of bound molecules of fa-Phe due to competition of other ligands for the available binding sites. For fa-Phe this percentage drops from 100%, when it is the only binding substance present in the mixture, to 30%, when all five competing ligands are present in the mixture. Also, for the fa-Phe-Phe ligand the trNOE intensity drops from 40% in mixture 2 to about 15% in mixture 5. This method provides us with the possibility to directly detect the competition of many ligands at the same time without the need to reference each of them to one standard. Thus, assessment of the relative binding strengths lets us evaluate the factors determining binding affinity.

The data shows that fa-Phe has the highest binding affinity of the fa derivatives. The phenyl group binding to the S1 subsite was shown to be significant for the

binding properties of inhibitors. Enalaprilat and lisinoprilat are ACE inhibitors that bind even tighter than captopril, which can also be accounted for by their binding of the phenyl group to the S1 subsite.[26,27] Studies of the enzyme kinetics of different fa substrates show that the substrate activity is largely influenced by the amino acid in position S1 with Phe > Leu > Ala > Gly.[17] Our results are in good general agreement with these data, since we found essentially the same order of binding affinities. The weaker binding affinity of fa-Phe-Phe does not fit this general picture but is rather likely caused by steric hindrance. We could also confirm the finding that the S1′ subsite has no mentionable importance toward binding strength. The relative binding strength of fa-Phe-Phe, fa-Gly-Leu-NH_2, and fa-Ala-Lys is not enhanced by the binding of the carboxy-terminal amino acid to the S1′ subsite.[9] Grobelny and Galardy have reported that optimal design of the ACE inhibitor is crucial for its binding properties. Too long or too short inhibitors do not allow for exact alignment of the essential functional groups and thus decrease binding affinity.[28] The S2 subsite has received only marginal attention, although one publication states that a large residue such as a benzoyl group is of advantage.[29] There is little data available about the importance of the fa group for binding affinity. The catalytic efficiency k_{cat}/K_M of ACE for the fa-Phe-Gly-Gly substrate is comparable to that of the natural substrates angiotensin I and bradykinin, with the turnover number k_{cat} being higher for fa-Phe-Gly-Gly. The data suggests that in fa-X-X derivatives both the fa fragment and the first amino acid are recognized by ACE. The fa group alone does not give rise to trNOE cross-peaks and, therefore, by itself does not bind to ACE, which was proven in a separate NOE experiment (data not shown).

trNOEs in the Presence of Captopril. In an experiment conducted separately, we added the inhibitor captopril, which has a high affinity for the S1′ and S2′ subsites, to a sample containing fa-Phe in the presence of ACE.[7] No trNOEs could be detected for captopril, since the dissociation kinetics for this inhibitor are too slow.[30] However, the experiment gave further strong evidence that fa-Phe specifically binds to the S1 and S2 subsites, since captopril did not change the trNOE cross-peak intensities of fa-Phe. Therefore, inhibition of this part of the receptor pocket does not interfere with binding of the fa-Phe ligand (Figure 8). On addition of an equimolar amount of fa-Phe-Gly-Gly to this mixture the substrate was no longer cleaved, as expected if inhibition by the tight-binding inhibitor captopril is complete. The intact substrate fa-Phe-Gly-Gly also revealed trNOE cross-peaks. The cross-peak intensities of fa-Phe and fa-Phe-Gly-Gly determined under the given conditions were equal. In other words, these two ligands occupy the same number of binding sites on the receptor. The high flexibility of the Gly-Gly dipeptide probably allows it to turn away from the protein having no adverse effect on the affinity of the ligand. We can therefore conclude that ACE binds the fa-Phe motif whether the S1′and S2′ subsites are blocked or not.

trNOEs in the Presence of a Large Excess of Dipeptides. A NOESY experiment was recorded after adding three dipeptides in a large molar excess (100-fold Asp-Ala, 120-fold Glu-Ala, and 80-fold Leu-Ala) to the mixture of the five fa derivatives in the presence of ACE. These dipeptides were shown to be weak, competitive, or mixed inhibitors of ACE by other groups (Asp-Ala, $K_D = 0.5 \times 10^{-3}$; Glu-Ala, $K_D = 1.3 \times 10^{-3}$; Leu-Ala, $K_D = 0.4 \times 10^{-3}$),[31] most likely interacting with the S1′ and S2′ subsites of the receptor. However, these dipeptides did not show trNOEs in the presence of ACE in NOE experiments conducted separately. In these separately conducted NOESY experiments they were added at an excess of ≈20:1, which is a reasonable excess for measuring trNOEs.[23] However, the addition of these dipeptides in a large excess to the mixture of the five fa derivatives in the presence of ACE led to a dramatic change of the trNOE intensities of the fa derivatives: fa-Trp and fa-Phe-Phe no longer bind to ACE, indicated by cancellation of trNOE cross-peaks (Figure 9), and the remaining ligands show a drastic change in distribution to the receptor site (Figure 10). One hypothesis explaining this cancellation of cross-peaks is the following: derivatives with a large amino acid in the S1 or S1′ subsite are hindered in their binding to the enzyme by the dipeptides which block the S1′ and S2′ subsites (Figure 9c). The fa derivative with the smallest amino acid in the S1 subsite, fa-Gly-Leu-NH_2, now reveals the strongest binding to ACE, which is probably due to the high flexibility of the Gly residue, allowing for easier adaptation to varying conditions in the receptor pocket; fa-Ala-Lys also has an increased binding constant compared to the mixture 5 shown in Figure 10a, which is probably due to the small amino acid in the S1 position. The effects of residues in the S1 position are now almost reversed compared to the case when no dipeptides are present. The binding affinities under these conditions are now in the order Gly > Phe ≈ Ala ≫Trp. The fact that fa-Trp no longer binds to ACE in the presence of the large excess of dipeptides indicates that the aromatic residue may be too large for the S1 binding site and must extend either into the S1′or the S2′ site.

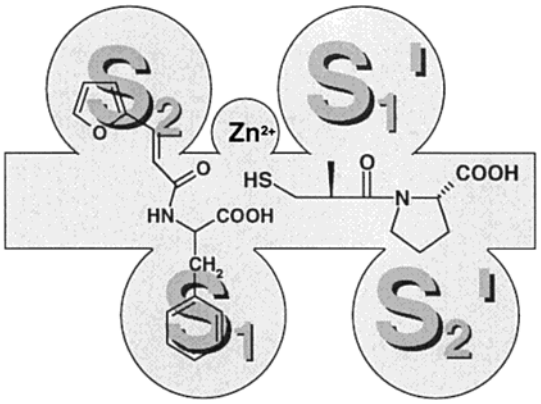


Figure 8. Proposed ACE binding site in the presence of fa-Phe and captopril. The binding of fa-Phe is not influenced by the high affinity inhibitor captopril, and therefore, it has the same binding affinity as in absence of the inhibitor.

Interestingly, only ligands interacting with the S1 and S2 subsites of ACE show trNOEs. We have tested other ligands presumably interacting exclusively with the S1′ and S2′ subsites, such as succinylproline, a precursor of captopril, as well as the above-mentioned dipeptides. Neither showed trNOE cross-peaks, although their

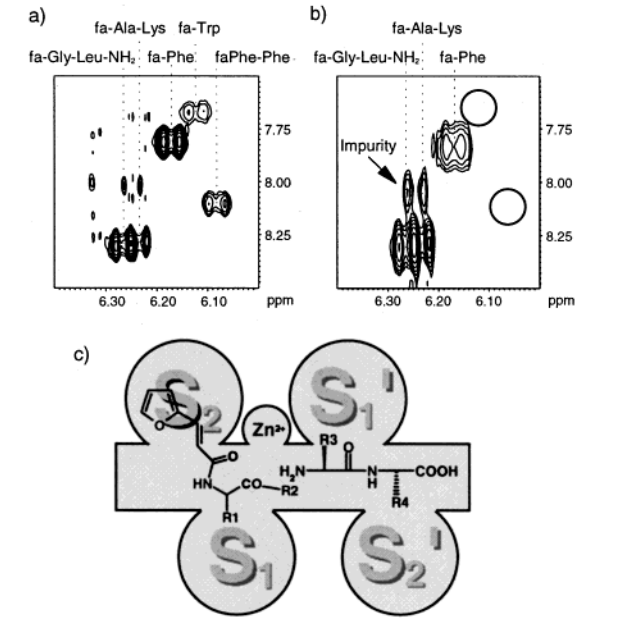


Figure 9. Cancellation of trNOE cross-peaks by addition of a large excess of dipeptides. Shown are the integrated cross-peaks prior to (a) and after (b) addition of the large excess of dipeptides. The region shown corresponds to the one indicated by the large circle in Figure 5. One can clearly observe the trNOEs of all five fa derivatives in spectrum a. The same region is displayed in spectrum b. The circles indicate the region where the cross-peaks from fa-Trp and fa-Phe-Phe have disappeared. One can also observe that the intensity of the fa-Phe cross-peak is significantly reduced. The explanation of the observed binding effects in the presence of fa derivatives and a large excess of dipeptides is shown in c. fa derivatives with a large group R1 or R2 have low affinities under these conditions because of interference with the dipeptides.

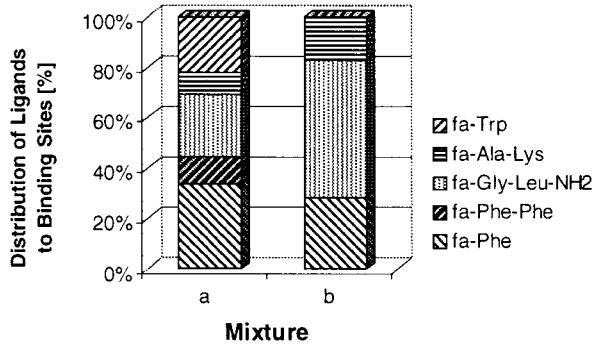


Figure 10. Distribution of the five fa derivatives, fa-Phe, fa-Phe-Phe, fa-Gly-Leu-NH_2, fa-Ala-Lys, and fa-Trp, to the available binding sites before (a) and after (b) the addition of a large excess of inhibitory dipeptides, i.e., Leu-Ala, Glu-Ala, and Asp-Ala, to mixture 5. The graph shows the large changes of binding affinities induced by the addition of the excess dipeptides. fa-Gly-Leu-NH_2 now has the highest affinity, whereas fa-Phe-Phe and fa-Trp no longer bind to the receptor showing the competition for the binding subsites. As a consequence, only fa derivatives with small and/or flexible amino acids in the first position still show binding, while the others, fa-Phe-Phe and fa-Trp, can no longer access the binding site (cf. the text).

binding constants lie well inside the regime normally observable by the trNOE approach. This may be due to a conformational rearrangement of the protein necessary to accommodate the ligand, thereby slowing down the binding kinetics. Therefore, due to the slow off rate constants, the trNOEs would not be observable.

Conclusions

In comparison to other currently available screening techniques, the bioaffinity NMR method has the advantage that identification of the binding compound may be detected even from a mixture of potential ligands, such as natural sources or combinatorial chemistry. The method proves to be especially powerful in absence of an X-ray structure of the protein cocrystallized with ligands. Comparing the relative binding strengths of many ligands at the same time allows a mapping of the binding site. Inclusion of large excess of other inhibitory substances can be used to further gain information of the relative orientation of the binding subsites. In addition, characterization of the ligand's 3D structure in the bound state may give further insight into the molecular basis of recognition.

Experimental Section

NMR Spectroscopy. ^{1}H NMR spectra were recorded at 500 MHz on a Bruker AVANCE DRX 500 spectrometer. The pulse sequence for the two-dimensional NOESY spectra included a filter to suppress zero quantum coherence.[32] The spectra for analyzing the mixtures were recorded with a mixing time of 100 ms in pure phase absorption mode using time proportional phase increment methods. The six spectra used for determining the buildup curve of fa-Phe in the presence of ACE were acquired with mixing times of 25, 50, 75, 100, 150, and 300 ms. Four spectra with mixing times of 300, 600, 900, and 1200 ms were acquired for analysis of the substrate fa-Phe-Gly-Gly in absence of ACE. Temperature in all spectra was set to 280 K. For the 2D spectra, water suppression was achieved by low-power preirradiation of the H_2O resonance during the 2 s relaxation delay and the mixing time. The spectral width was set to 5000 Hz. A total of 32−64 transients were recorded for each of the 128 increments with 4K data points. After Fourier transformation 4K × 1K data matrixes were obtained. The 1D spectra were recorded with a standard watergate pulse sequence from the Bruker pulse program library. Processing and integration were performed on Silicon Graphics Workstations with Bruker Software (XWINNMR 1.3 and Aurelia 2.1.3). Determination of the line width at half-height of the 1D ^{1}H NMR spectra was achieved by fitting the f3 resonance of fa-Phe to a Lorentzian model using Microcal Software (Origin 6.0).

Rabbit lung angiotensin-converting enzyme (MW = 130 kDa) and the fa derivatives were purchased from Sigma, and the dipeptides were from Bachem. A 5 mg (40 nmol) portion of ACE was dissolved in 0.7 mL of 90% H_2O/10% D_2O of a 10 mM phosphate buffer (pH 7) containing 20 mM NaCl, 10 μM $ZnCl_2$, and 0.02% NaN_3. The fa derivatives (fa-Phe, fa-Phe-Phe, fa-Gly-Leu-NH_2, fa-Ala-Lys and fa-Trp) were added in 15−30-fold excess (0.6−1.2 μmol). The dipeptides were added in 80−100 fold excess (3.2−4 μmol).

ACE Activity Tests. A photometric assay for activity was performed by monitoring the decrease in absorption caused by enzymatic hydrolysis of fa-Phe-Gly-Gly.[16] ACE activity was measured over a 5 min period at 340 nm and 37 °C. These measurements confirmed that activity remained stable at NMR measuring conditions (280 K, pH 7) during the experiments.

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