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The probable conformation of substrates recognized by dipeptidyl-peptidase IV and some aspects of the catalytic mechanism derived from theoretical investigations

W. Brandt[a,*], T. Lehmann[a], T. Hofmann[a], R.L. Schowen[b] and A. Barth[a]

[a]*Martin-Luther-University Halle-Wittenberg, Institute of Biochemistry, Weinbergweg 16a, Halle/Saale, Germany*
[b]*Departments of Chemistry and Biochemistry, University of Kansas, Lawrence, KS 66045-0046, U.S.A.*

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SUMMARY

By theoretical conformational investigations of substrates and nonsubstrates of the enzyme dipeptidyl-peptidase IV (DP IV) as well as dipeptide-esters using the ECEPP83 method we determined the structure of peptides recognized and cleaved by the enzyme. From a comparison of all possible structures for the substrates with conformations not possible in nonsubstrates we concluded that a single conformation explains substrate specificities of DP IV. This conformation is characterized by the following dihedral angles: $\Psi_1 = 85°$, $\omega_1 = 180°$, $\Phi_2 = -75°$, $\Psi_2 = 80°$, and $\omega_2 = 180°$. The conclusions were supported by comparisons of molecular electrostatic potentials calculated with the molecular graphics program HAMOG.

INTRODUCTION

Dipeptidyl-peptidase IV (DP IV) is a serine peptidase of broad medical and biochemical significance, which sequentially removes the N-terminal dipeptide of a peptide chain whenever a proline or alanine residue is present in the P_1-position (scissile residue) of the substrate [1–11]. Peptides with Pro at the P'_1-position (C-terminal of the scissile residue) are not hydrolyzed. Any essential amino acid can occupy the P_2-position (N-terminal from the scissile residue) as long as the N-terminus is unprotected and the N-terminal amino function is protonated. The stereospecificity for the amino acid in P_2-position depends on the amino acid residue in P_1-position. If Pro is in the P_1-position then the cleavage of the substrates takes place only when L-amino acids are existent in P_2-

* To whom correspondence should be addressed.

position. In this case the P_2-position is stereospecific in regard to the enzymatic hydrolysis of the substrates. If the amino acid residue in P_1-position is Ala, then DP IV hydrolyzes peptides with both D- and L-amino acids at P_2. From kinetic investigations it is known that at the pH optimum the rate-limiting step for the aminoacyl-proline-4-nitroanilides is the deacylation and for the aminoacyl-alanine-4-nitroanilides the acylation reaction [12, 13].

The aim of the present theoretical investigation was to study the role of substrate conformation in the mechanism of substrate cleavage and specificity of DP IV. Since an X-ray structure of DP IV could not be obtained up till now we proceed this way to get some structure information. With some exceptions, most previous models of substrate hydrolysis by serine proteases have tended to describe the amide cleaving mechanism without regard for a possible interaction between the catalytic action and the specificity for substrates [12–24]. In view of the high substrate specificity of DP IV, we decided to study the conformations of several substrates and 'nonsubstrates', hoping to find one structure which could explain, on the one hand, the substrate specificity of DP IV and, on the other hand, the first important step of the hydrolysis mechanism of DP IV.

METHODS

We used the ECEPP83-method [25] (ECEPP – Empirical Conformational Energy Program for Peptides) for the calculation of the relative conformational energy of the peptides. The calculation and comparison of molecular electrostatic potentials were performed with the molecular graphics program HAMOG [26] developed by ourselves. Within HAMOG an electronegativity equalization method [28, 29] for the calculation of point charges is applied for qualitative comparisons of molecular electrostatic potentials. Our conformational investigations are restricted to tripeptide units since all experimental results relate to the first 3 amino-acid residues, starting from the N-terminus [1–11]. The results presented here are those for the N-terminal protonated peptides if not otherwise stated. If the charge is excluded, there is no essential difference in the results. The influence of the C-terminal amino acid residues is modeled by a methyl-amide group.

For each molecule, we calculated many conformational maps to assure that all stable conformations of the compound would be found. We varied the dihedral angles Φ (N-C_α) and Ψ (C_α-C') for each residue, and also considered cis- and trans-conformations of amide linkages. Two dihedral angles were varied in steps of 20° over the whole conformational space, fixing all other angles. The minima thus located were refined by optimization of all dihedral angles.

RESULTS AND DISCUSSION

Taking note of the fact that DP IV recognizes substrates whenever a proline or alanine residue is in the P_1-position of a peptide, we first compared the conformations of a series of such compounds. The dihedral angle of proline is relatively rigid because of the 5-membered pyrrolidine ring; it varies between $-40°$ and $-80°$ [29] and is fixed within the ECEPP83 program at values of $-68°$ (Pro-up) and $-75°$ (Pro-down).

Conformational investigations of *N*-acetyl-*N'*-methylprolylamide, carried out by us with both Pro-up and Pro-down, gave the same results as those of Chuman et al. [30]. There are 5 stable conformations for either Pro-up and Pro-down structures of the pyrrolidine ring. Since the conformational dependence on Φ is only slight we will present only those results calculated with a value of

$\Phi = -75°$. We have also made calculations for all molecules, using a value of $\Phi = -68°$, and compared the results with the Pro-down structures. There are no essential differences in conformational behavior in the two cases.

CONFORMATIONAL INVESTIGATIONS OF DIPEPTIDE SUBSTRATES

Prolyl-proline-methylamide is the dipeptide with the least conformational degrees of freedom in comparison to all other substrates studied. If two proline residues are present in the P_1- and P_2-positions and not in the P_1'-position, the enzyme accepts the peptide as a substrate very well [1–13]. We have calculated all low-energy conformations of the cation of prolyl-proline-methylamide. In Table 1 are listed the dihedral angles and corresponding energy values for all refined conformational minima, both for the cis-conformation ($\omega = 0°$) and the trans-conformation ($\omega = 180°$). Since this molecule is a substrate for DP IV, we conclude that one of these 10 conformations should be a structure which is accepted by the enzyme. In contrast to prolyl-proline the dihedral angles Φ of alanyl-alanine-methylamide are flexible. The side-chain conformation must also be considered, but the calculations showed that the side-chain angles are nearly constant at values of 60°. Since the proline substrates showed a dihedral angle Φ of $-75°$, we examined the alanine series for energetically stable conformations with nearly the same value. In Table 2 are listed all stable conformations found with trans- or cis-conformations for the peptide bond between the two residues. The most stable conformation found is nearly identical with the conformation in the crystal structure of Ala-Ala-4-nitroanilide [31]. The results shown in Table 2 reveal that there are indeed some conformations where Φ is equal to $-75°$. This result is an indication that the proline substrates as well as the alanine substrates are recognized by the enzyme in the same manner which is an absolute prerequisite for all further considerations.

These conclusions can be supported by comparison of the molecular electrostatic potentials (Figs. 1–3). Using the molecular graphics program HAMOG [26] the molecular electrostatic potentials were calculated on the 1.7-fold van der Waals surface of the molecules and represented as

TABLE 1
CONFORMATIONS OF LOW ENERGY OF THE PROLYL-PROLINE-METHYLAMIDE (CATION)

No.	Dihedral angles (in deg)							Energy (kJ/mol)
	Φ_1	Ψ_1	ω_1	Φ_2	Ψ_2	ω_2	Φ_3	
1	−13	170	173	−75	173	180	60	0.0
2	−13	170	174	−75	78	178	61	3.8
3	−13	170	176	−75	−49	180	60	8.0
4	−13	82	180	−75	170	180	60	8.8
5	−13	82	179	−75	79	178	61	10.5
6	−13	82	180	−75	−20	180	60	13.8
7	−13	165	−9	−75	177	179	60	8.4
8	−13	166	−9	−75	−50	180	60	8.8
9	−13	82	17	−75	155	180	61	26.0
10	−13	81	16	−75	−57	−178	60	27.6

TABLE 2
CONFORMATIONS OF LOW ENERGY OF THE ALANYL-ALANINE-METHYLAMIDE (CATION)

No.	Dihedral angles (in deg)									Energy (kJ/mol)
	Φ_1	Ψ_1	ω_1	Φ_2	Ψ_2	ω_2	Φ_3	χ^1_1	χ^1_2	
1	10	165	179	−155	159	180	60	180	179	0.0
2[a]	8	166	178	−71	155	180	60	−179	−178	0.4
3[a]	11	164	180	−83	86	179	61	180	180	3.3
4[a]	10	164	180	−73	−40	180	60	180	−179	4.2
5	10	164	180	−153	48	180	60	180	−179	6.0
6	9	165	179	−158	−58	179	60	−179	172	8.4
7	8	165	179	54	58	180	60	180	−174	10.0
8	−13	−55	174	−73	156	180	60	180	−177	10.9
9[a]	−59	(85)[b]	179	−75	152	180	60	178	−177	11.3
10	14	160	−179	63	−177	179	61	179	−157	12.1
11	−21	−53	179	−153	163	180	60	−179	180	12.6
12[a]	−11	(85)[b]	180	−73	(85)[b]	177	60	180	180	12.6
13	38	−65	179	−153	−163	180	60	169	180	13.0
14[a]	−59	(85)[b]	−179	−75	−38	180	60	−179	180	15.1
15	−20	−56	179	−159	−59	180	60	180	171	23.9
16	−27	−57	180	−74	−43	180	60	180	−179	24.3
17	55	−67	179	−74	−43	180	60	169	−179	24.7

[a] Conformations which correspond to structures of Pro-Pro.

[b] ( )Dihedral angles are fixed. There are some further minima which have an energy higher than 25 kJ/mol. Conformations with cis-peptide bonds have an energy higher than 50 kJ/mol.

the interaction energy with a proton. The factor 1.7 was chosen to ensure the physical importance up to a distance not smaller than 3 Å to the next atom if using point charge representations for

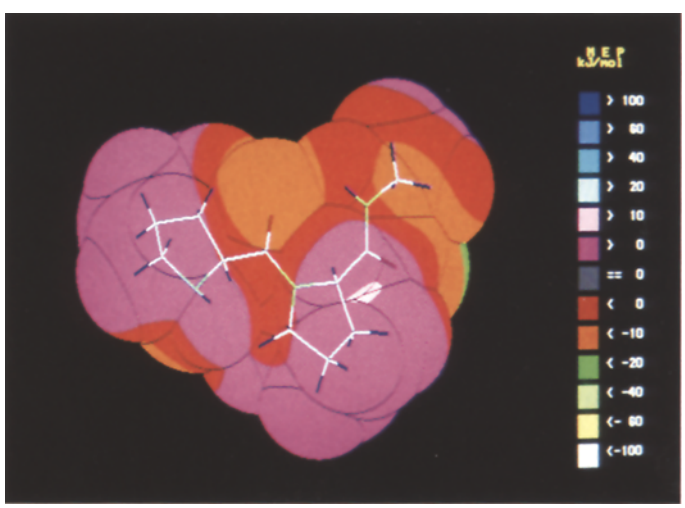

Fig. 1. Molecular electrostatic potential of Pro-Pro-$NHCH_3$ on its 1.7-fold van der Waals surface. The colors correspond to the energy of an electrostatic interaction with a proton.

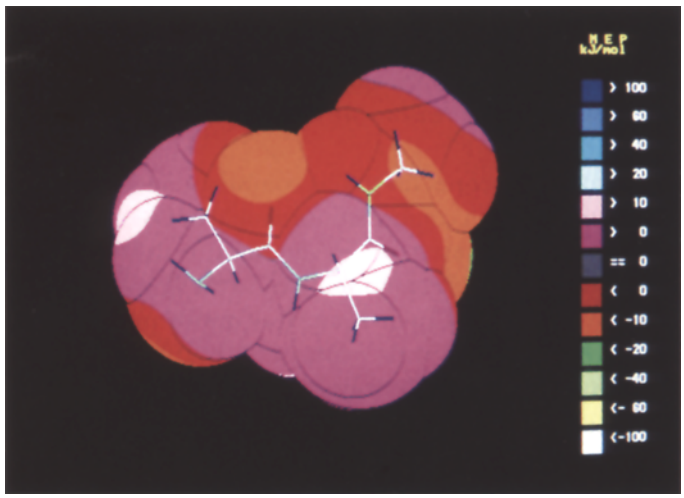

Fig. 2. Molecular electrostatic potential of Ala-Ala-$NHCH_3$ on the 1.7-fold van der Waals surface of the molecule.

such calculations. The comparison of the two molecular electrostatic potentials (Fig. 3) shows that the interaction behavior of the alanine and proline substrates with the enzyme should be nearly identical. Identical signs of the potential values of both molecules are predominant (red and purple areas).

Furthermore, we can assume that the difference in the rate-limiting step between the proline and alanine substrates is caused by the much higher flexibility and conformational variety of the

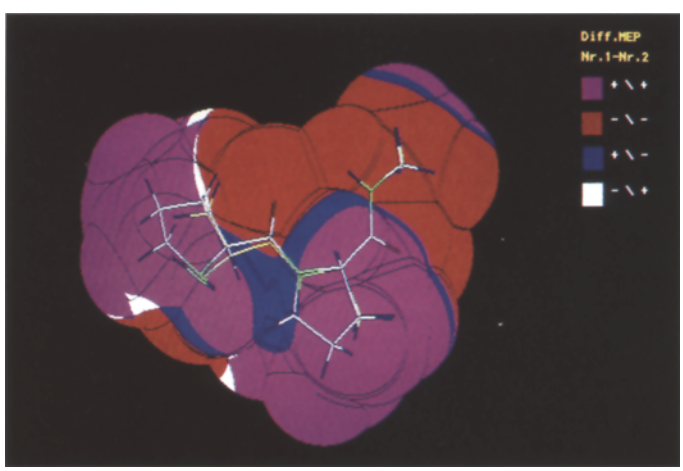

Fig. 3. The comparison of the molecular electrostatic potentials shown in Figs. 1 and 2 on the outer 1.7-fold van der Waals surface formed by the two superimposed molecules (RMS = 0.066 Å) with respect to their sign. Red colors, for instance, indicate negative potential in this position for both molecules. The small blue and white areas represent a difference between the two electrostatic potentials.

alanine substrates. Since the statistical weight of probability of the occurrence of the right conformation to be recognized by DP IV is much lower for the alanine substrates than for the proline substrates (compare the number of the conformations in Tables 1 and 2) the hypothesis seems to be allowed that this is at least one of the reasons why in alanine substrates the formation of the acylenzyme is the rate-limiting step.

Since the relative conformational energies for the cis-conformations are higher than 50 kJ/mol, it seems reasonable to conclude that such conformations should not be relevant for the hydrolysis mechanism of DP IV. All experimental results are in agreement with this conclusion [32]. Therefore at this point, we restricted the possible conformations to the first 6 given in Table 1. Similar conformations of the Ala-Ala substrate in Table 2 are labeled with an [a]. It was, however, impossible to obtain minimum-energy structures with values of Ψ_1 near 85°. To compare the relevant structures of Ala-Ala substrates with those of Pro-Pro substrates, we calculated a conformational map for Ψ_1 and Ψ_2 at fixed Φ_2 (−75°). It emerged that certain combinations of dihedral angles (minima 4–6 of Table 1) correspond to relatively low-energy regions of the potential surface. In the next step, we kept the value of Ψ_1 at 85° and initiated further optimization of all other dihedral angles. The results in Table 2 show that the energy is only slightly higher than the corresponding true minima for refinement of all dihedral angles. The maximal relative energy difference with respect to the most stable conformation is 15 kJ/mol. It would seem quite possible that energy of this magnitude could be obtained from the interaction of the substrate with the enzyme.

The interesting result that stable conformations were found with dihedral angles corresponding to the first 6 structures listed for Pro-Pro substrates provokes the question whether or not other dipeptides have similar features. Results concerning some other calculated dipeptides have been published [33]. The conclusions are the same as for Ala-Ala substrates and Pro-Pro substrates, re-

TABLE 3
CONFORMATIONS OF LOW ENERGY OF THE D-ALANYL-ALANINE-METHYLAMIDE (CATION)

No.	Dihedral angles (in deg)									Energy (kJ/mol)
	Φ_1	Ψ_1	ω_1	Φ_2	Ψ_2	ω_2	Φ_3	$\chi^1{}_1$	$\chi^1{}_2$	
1	−10	−164	−179	−156	160	180	60	180	179	0.0
2[a]	−14	−161	179	−70	155	180	60	−179	−178	0.0
3[a]	−10	−164	−179	−84	(80)[b]	179	60	180	−179	4.0
4	−8	−165	−179	−155	50	180	60	180	180	5.4
5[a]	−8	−165	−179	−74	−41	180	60	179	−179	5.4
6	−11	−164	180	−159	−58	179	60	180	172	7.5
7	−11	−163	180	54	57	180	60	180	−174	9.2
8	−8	−167	−178	63	−177	178	61	179	−157	13.0
9	−8	54	−179	−157	158	180	60	179	179	13.0
10[a]	22	55	179	−74	151	180	60	179	−178	16.3
11[a]	21	54	−179	−84	83	179	61	180	180	17.6
12[a]	21	56	−179	−73	−38	180	60	180	−179	21.4
13	20	55	−176	63	−174	178	61	180	−156	24.7

[a] Conformations which correspond to structures of Pro-Pro.
[b] ( ) Dihedral angle is fixed.

TABLE 4
CONFORMATIONS OF LOW ENERGY OF THE D-ALANYL-PROLINE-METHYLAMIDE (CATION)

No.	Dihedral angles (in deg)								Energy (kJ/mol)
	Φ_1	Ψ_1	ω_1	Φ_2	Ψ_2	ω_2	Φ_3	$\chi^1{}_1$	
1[a]	−29	−148	180	−75	173	180	60	−173	0.0
2[a]	−29	−148	180	−75	81	178	61	−172	4.2
3	9	−74	166	−75	173	180	60	179	4.6
4[a]	−27	−150	−177	−75	−51	180	60	−172	8.4
5	6	−74	172	−75	81	178	60	179	12.1
6	6	−75	173	−75	−52	180	60	179	20.9

[a] Conformations which correspond to structures of Pro-Pro.

spectively. The most stable conformation of the Ala-Pro substrate is very similar to the crystal structure of Ala-Pro-4-nitroanilide [34]. The results of our conformational investigations of these dipeptides thus tend to confirm the view that one of the stable trans-conformations of the Pro-Pro substrate must be important for hydrolysis by DP IV. We next investigated the basis for the observation that D-Ala-Ala substrates are slowly hydrolyzed by DP IV, while D-Ala-Pro substrates are not hydrolyzed at all.

We calculated conformational maps with a fixed dihedral angle of $\Phi_2 = -75°$ for both types of substrates. All stable conformations are presented in Tables 3 and 4. If the conformational properties of the two molecules are compared, remarkable differences become obvious. The D-Ala-Ala substrate has 3 true minima for Ψ_1 between 54° and 66° (minima 10–12 in Table 3). The energy expense to obtain a conformation with $\Psi_1 = 80°$ analogous to that of the Pro-Pro substrate, is only 6 kJ/mol. In contrast, the D-Ala-Pro substrate cannot attain a similar conformation. Very high energies for conformations with Ψ_1 near 85° are caused by steric hindrance between the side chain of the D-Ala residue and the methylene group of the C_δ atom of proline, which is not present in the case of D-Ala-Ala (Fig. 4). Conformations with values of Ψ_1 near 160° occur in both molecules. Therefore, because conformations with Ψ_1 near 85° are impossible for D-Ala-Pro but possible for D-Ala-Ala, and since D-Ala-Pro is not hydrolyzed by DP IV, we conclude that their different conformational behavior is responsible for the different enzymatic activity. If this statement is indeed true it leads to the assumption that the absolute stereospecificity for L-aminoacyl-prolyl-4-nitroanilides with respect to D-aminoacyl-prolyl-4-nitroanilides is shown in the state of the Michaelis–Menten complex. Assuming that the different activity is caused by sterical hindrance of interactions with the enzyme (assuming that the conformations with Ψ_1 about 160° are recognized), it would not be possible to explain the different behavior. The logical consequence of this conclusion is that one of the 3 conformations with Ψ_1 near 85° is the conformation which is accepted by DP IV. In other words, it is now possible to exclude all conformations except the 3 which correspond to minima 4–6 of Table 1. These 3 conformations are shown in Fig. 5 for the prolyl-proline-methylamide (cation). The dihedral angle Ψ_1 near 85°, which is common for all substrates, causes a nearly perpendicular orientation of the two pyrrolidine rings. The energy differences among the 3 conformations result only from the 3 different values of the dihedral angle Ψ_2.

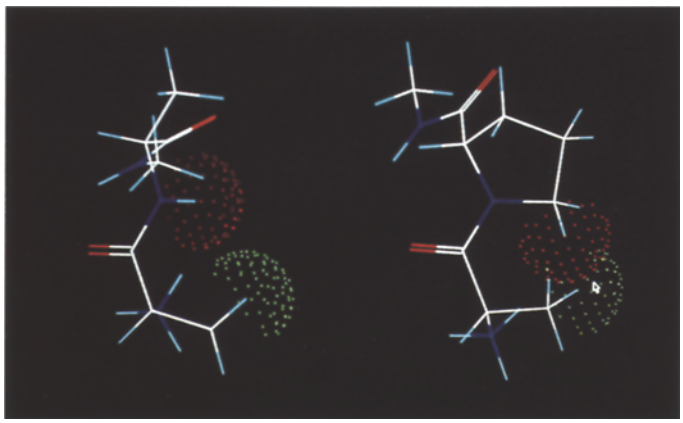

Fig. 4. Conformation of D-Ala-Ala (left) corresponding to minimum 11 of Table 3 and conformation of D-Ala-Pro (right) which is energetically impossible as indicated by the overlap of the van der Waals surfaces because of the steric hindrance of the D-Ala side chain with the pyrrolidine ring.

In our investigations of the β-casomorphins [35], we calculated, independently of our current results, many structures of β-casomorphin-4-amid (morphiceptin = Tyr-Pro-Phe-Pro-NH_2) which is also a substrate of DP IV. We found for the tyrosyl-proline-methylamide as well as for the phenylalanyl-proline-methylamide conformations which correspond to the 3 structures proposed here as essential for substrates of DP IV. These examples support our assumption, that all essential amino acid residues in the P_2-position can attain the proposed 3 conformations in agreement with the experimental facts.

Which of the 3 structures is the one actually favored by the enzyme cannot be determined by calculations on other dipeptide substrates. Therefore we examined tripeptide substrates.

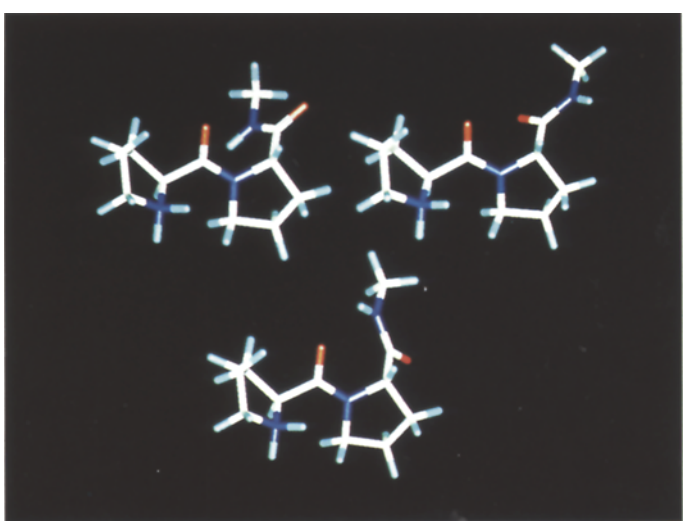

Fig. 5. The three stable conformations of Pro-Pro with respect to the dihedral angle Ψ_2 (left 20°, middle 80° and right 170°)

CONFORMATIONAL INVESTIGATIONS OF TRIPEPTIDES

For all tripeptide substrates, we calculated conformational maps for the dihedral angles Φ_3 and Ψ_3 by fixing the other dihedral angles at values corresponding to all minima found for dipeptide substrates. All minima found on the maps were then refined.

The results obtained for prolyl-prolyl-glycine-methylamide (cation) are listed in Table 5. As we expected, the conformations of the first two amino acid residues are only slightly changed from those in the corresponding dipeptide substrates (see Table 1). Even the relative energy graduation is nearly the same. Comparing these conformations and their corresponding relative energies with other tripeptides [33] modified in position P_2, it is interesting to note that the relative energies of the possible relevant conformation of substrates with proline in P_2-position are essentially lower than, for instance, in the Gly-Pro-Gly substrate. This behavior is probably caused by weak repulsive interactions between the C_β-methylene group of the N-terminal proline residue and the C_γ-methylene group of the second proline residue within its most stable conformation ($\Psi_1 = 170°$) which does not occur in the conformation with $\Psi_1 = 80°$ and in the most stable conformations of the other molecules. Therefore, the energy loss is smaller for Pro-Pro if the hydrogen bond between the N-terminus and the carbonyl group disappears by changing the dihedral angle Ψ_1 from 170° to 80°. Maybe this is one of the reasons, why for substrates with proline in P_2-position the most reactive substrate results.

As an example for the alanine series, we considered the tripeptide glycyl-alanyl-glycine-methylamide (cation). The results are shown in Table 6. We found, as in the case of the dipeptide substrates, conformations with a value of the dihedral angle Φ_2 near $-75°$ and for Ψ_1 values of near 85°, which are not true minima but only somewhat higher in energy than the corresponding true minima. Thus our conclusions from above remain valid.

Prolyl-prolyl-proline is not hydrolyzed by DP IV. Our calculations for this compound produce

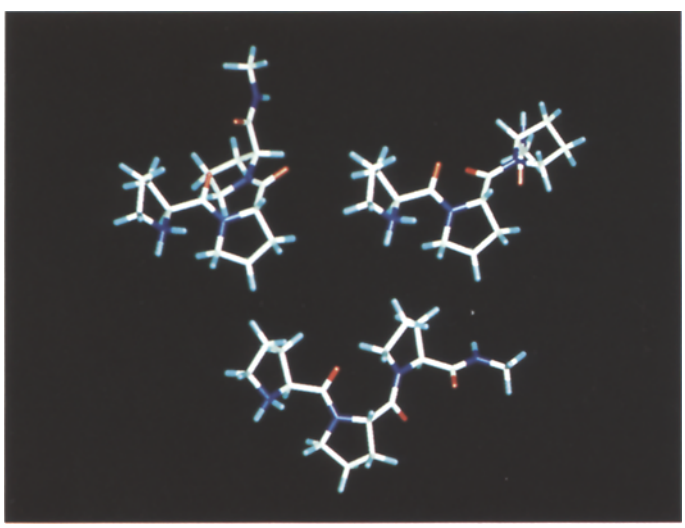

Fig. 6. In comparison to Fig. 3 the analogous conformations for the tripeptide Pro-Pro-Pro. The left ($\Psi_2 = -20°$) and middle ($\Psi_2 = 80°$) are sterically impossible.

TABLE 5
CONFORMATIONS OF LOW ENERGY OF PROLYL-PROLYL-GLYCINE-METHYLAMIDE (CATION)

No.	Dihedral angles (in deg)										Energy (kJ/mol)
	Φ_1	Ψ_1	ω_1	Φ_2	Ψ_2	ω_2	Φ_3	Ψ_3	ω_3	Φ_4	
1	−13	170	173	−75	173	180	−179	179	180	60	0.0
2	−13	82	180	−75	−16	179	−171	−178	180	60	7.1
3	−13	83	178	−75	76	−179	81	−82	−179	59	7.9
4	−13	83	177	−75	73	180	168	175	180	60	7.9
5	−13	82	180	−75	171	180	−179	180	180	60	8.4
6	−13	82	180	−75	170	180	−83	78	179	60	9.6
7	−13	82	180	−75	169	180	83	−77	−179	60	9.6
8	−13	81	180	−75	97	−176	82	29	180	63	9.6
9	−13	82	180	−75	−21	178	−83	80	179	61	10.4
10	−13	82	179	−75	78	180	−83	78	180	60	10.4
11	−13	82	180	−75	171	180	−72	−42	180	60	10.9
12	−13	82	180	−75	164	−179	70	38	180	60	11.3
13	−13	82	178	−75	75	180	176	61	180	60	11.3
14	−13	82	180	−75	79	−179	157	−40	180	61	11.3
15	−13	82	180	−75	−19	180	83	−78	180	60	12.1
16	−13	82	180	−75	171	180	−169	53	180	60	12.1
17	−13	82	180	−75	171	180	173	−55	180	60	12.5
18	−13	82	180	−75	−15	175	−120	34	180	61	13.4
19	−13	82	179	−75	78	179	−73	−38	180	60	13.4
20	−13	82	180	−75	−15	178	−76	−25	180	60	14.2
21	−13	82	180	−75	−20	179	170	−58	180	60	15.5
22	−13	82	180	−75	−21	−179	75	32	180	60	15.5

only the 3 conformations listed in Table 7. These structures differ only with respect to the dihedral angle Ψ_3. For the dihedral angle Ψ_2, only values of nearly 167° are possible. Conformations with values of Ψ_2 about −20° and 80° are impossible (Fig. 6). The replacement of the hydrogen of the dipeptide substrates by the CH_2-group of the third pyrrolidine ring prevents by steric hindrance the formation of hydrogen bonds, both to the first carbonyl oxygen atom ($\Psi_2 = 80°$) and to the nitrogen atom of the second pyrrolidine ring ($\Psi_2 = -20°$).

It seems possible that this conformational difference, significant with respect to all other molecules with any amino acid residue other than proline in the P_1'-position, is of importance for the substrate specificity of DP IV. If this hypothesis is indeed true, it leads immediately to the consequence that one of the two unattainable conformations is the essential one for the DP IV. Therefore we believe, we can at this point restrict the possible conformations of substrates recognized by the enzyme to these two ($\Psi_2 = 80°$ or $\Psi_2 = -20°$).

CONFORMATIONAL INVESTIGATIONS OF DIPEPTIDE-ETHYL ESTERS

During the first steps of substrate hydrolysis by DP IV a tetrahedral intermediate is formed by interaction of the side chain of a serine residue of the enzyme with the carbonyl group of the bond

TABLE 6
CONFORMATIONS OF LOW ENERGY OF THE GLYCYL-ALANYL-GLYCINE-METHYLAMIDE (CATION)

No.	Dihedral angles[a] (in deg)								Energy (kJ/mol)
	Φ_1	Ψ_1	ω_1	Φ_2	Ψ_2	ω_2	Φ_3	Ψ_3	
1	40	−138	171	−60	−43	170	−91	155	0.0
2	−34	142	−170	48	54	−171	84	−149	2.1
3	−13	−161	175	−62	−43	173	−85	153	3.1
4	−53	(80)[a]	179	−83	79	−178	76	162	15.2
5	−53	(80)[a]	179	−68	113	−178	78	25	15.3
6	−53	(80)[a]	180	−74	−32	179	−172	−178	16.0
7	−53	(80)[a]	179	−73	151	180	−179	−179	16.0
8	−53	(80)[a]	179	−69	148	−179	83	−76	16.6
9	−52	(80)[a]	−179	−83	(80)	179	81	−82	16.8
10	−53	(80)[a]	−179	−84	(80)	179	175	177	17.0
11	−53	(80)[a]	180	−68	−41	178	−82	79	17.2
12	−53	(80)[a]	179	−72	150	−179	−82	78	17.2
13	−53	(80)[a]	179	−74	154	179	−74	161	17.9
14	−53	(80)[a]	−179	−84	(80)	179	−83	77	18.1
15	−53	(80)[a]	179	−68	−44	176	−84	−150	18.4
16	−53	(80)[a]	179	−73	151	180	−73	−39	19.1
17	−53	(80)[a]	179	−73	149	180	168	−52	19.5
18	−54	(80)[a]	179	−73	149	−179	−172	54	19.9
19	−53	(80)[a]	−179	−73	−38	180	83	−79	19.9

[a] ( ) Dihedral angles are fixed. ω_3 is nearly 180° and $\chi^1{}_2$ and Φ_4 have values of 60°.

to be cleaved. This tetrahedral intermediate decomposes into a stable acyl enzyme, with liberation of the rest of the peptide.

Our basic assumptions are that the reaction barriers for the formation and decomposition of the tetrahedral intermediate are relatively low in energy and that the dipeptide ester part of acyl enzyme should exist in a relatively stable conformation.

Therefore we calculated the conformations of some dipeptide-ethyl esters, the ethyl group being chosen to model the serine side chain of the enzyme. Starting from the stable conformations found for the dipeptide substrates, we calculated all possible structures of the Gly-Pro, Ala-Pro-, Ala-Ala- and D-Ala-Ala-ethylesters (see for example Table 8). The results obtained for all compounds

TABLE 7
CONFORMATIONS OF LOW ENERGY OF PROLYL-PROLYL-PROLINE-METHYLAMIDE (CATION)

No.	Dihedral angles (in deg)										Energy (kJ/mol)
	Φ_1	Ψ_1	ω_1	Φ_2	Ψ_2	ω_2	Φ_3	Ψ_3	ω_3	Φ_4	
1	−13	82	180	−75	167	171	−75	75	179	60	0.0
2	−13	81	180	−75	168	171	−75	170	180	60	0.4
3	−13	82	180	−75	167	173	−75	−44	180	60	4.6

TABLE 8
CONFORMATIONS OF THE GLYCYL-PROLINE-ETHYLESTER (CATION)

No.	Dihedral angles (in deg)								Energy (kJ/mol)
	Φ_1	Ψ_1	ω_1	Φ_2	Ψ_2	ω_2	Φ_3	Ψ_3	
1	−3	−175	178	−75	−176	180	180	60	0.0
2	−3	−175	178	−75	−177	−170	74	54	7.5
3	−11	84	180	−75	−8	−170	74	53	1.7
4	−10	84	179	−75	171	180	180	60	16.3
5	−11	84	180	−75	0	178	−179	60	18.0
6	−10	84	180	−75	159	−167	72	53	23.0
7	−10	84	179	−75	171	170	−74	67	23.4
8	−10	84	−179	−75	−5	165	−72	67	23.9

indicate that there are only conformations with Ψ_2 near 170° and Ψ_2 near −20° (Fig. 7). The two structures in which Ψ_2 differs by about 180°, arise because of repulsive electrostatic interactions between the oxygen atoms of the ester group and the carbonyl oxygen atom of the first peptide bond. For this reason a conformation with $\Psi_2 = 80°$ is impossible and has very high energy.

What can we conclude from these results? As we stated above, the reaction for forming the acyl enzyme should be possible without a high reaction barrier. If we assume that the conformation within the recognition complex is not very far away from the structure of the transition state and the stable intermediate (acyl enzyme), we have to look for a conformation of the substrates which is favored to form a recognition complex with a preformed structure of the stable intermediate. Because of the very special substrate specificity of the dipeptidyl peptidase IV it seems to be possible that the catalytic mechanism is different from other known serine proteases. The amino-acid sequence of the dipeptidyl peptidase IV isolated from rat liver [36] shows no homologies to the other members of this enzyme family, therefore a comparison with the active sites of such enzymes should not be suitable. The only way to get further information by theoretical investigations with regard to the recognition structure and first hypothetical insights in a probable reaction mechanism at this state of knowledge seems to be to compare the conformations of the substrates and of their ethyl esters.

The tetrahedral intermediate can be formed in principle by attack of the serine from either of the two faces of the amide linkage. Therefore we have to consider the two conformations already determined to be important, and how it is possible to form the tetrahedral intermediate and then the acyl enzyme.

First, we consider the conformation with $\Psi_2 = -20°$. The serine oxygen atom may attack the peptide bond from a direction which can be described by means of the dihedral angle to be either $\Psi_2 = 70°$ or $\Psi_2 = -110°$ (perpendicular with respect to the peptide bond to be cleaved). In the first case a repulsive interaction of the serine oxygen with the carbonyl oxygen of the first peptide bond would occur as shown in Fig. 8 and concluded from our calculations on the ethyl esters. This process should therefore be very unlikely. In the second case (direction of attack $\Psi_2 = -110°$), the transformation of the tetrahedral intermediate into one of the calculated stable ester conformations should require passage over high-energy barriers. Either the carbonyl oxygen atom has to

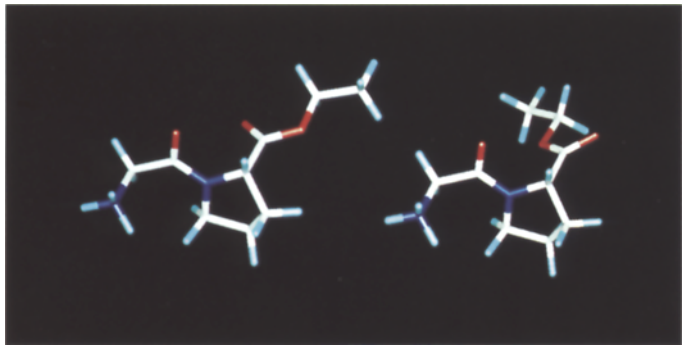

Fig. 7. The only two possible conformations with respect to Ψ_2 for Gly-Pro-ethylester.

move 180° along a path of strong repulsive interaction with the oxygen atom of the first peptide bond (to attain a planar ester group with an ester conformation of $\Psi_2 = 170°$, Figs. 5 and 7) or the serine side chain (ethyl group) has to twist around the carbonyl carbon atom through a sterically hindered region to reach the ester conformation with $\Psi_2 = -20°$. Both cases seem to be very unlikely even when the tetrahedral intermediate is stabilized by the interaction with the active site of the enzyme. The problems especially of repulsive electrostatic interactions with the first carbonyl oxygen atom remain unchanged.

The conditions are much better for the starting conformation with $\Psi_2 = 80°$. The serine may attack from a direction of $\Psi_2 = -20°$ or 170°. In both cases, the attacking serine oxygen atom is just in the position required for the stable ester conformation previously found (compare Figs. 5, 7 and 8). The carbonyl oxygen atom could move during the decomposition of the tetrahedral intermediate to any angle of about 90° without any electrostatic or steric hindrance in either case.

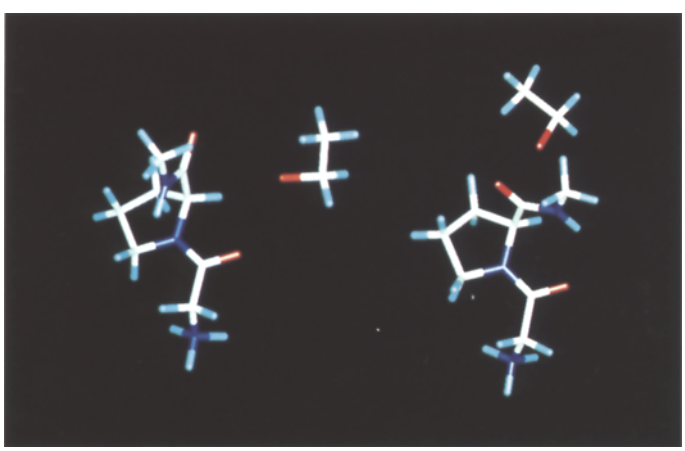

Fig. 8. The direction of attack (left) of a serine oxygen atom for a substrate conformation with $\Psi_2 = -20°$ is impossible because of the electrostatic repulsion between the two oxygen atoms (red), whereas it seems to be possible (right) for a substrate conformation with $\Psi_2 = 80°$ without any steric hindrance or electrostatic repulsion. The oxy-ethyl group has been chosen to mimic the serine side chain.

If an oxygen atom is favored to interact with the carbonyl carbon atom of the peptide bond to be cleaved, a positive molecular electrostatic potential should be assumed in the direction of attack. From this a further argument for our conclusions drawn above can be derived. Thus, it is known that the free dipeptides are competitive inhibitors of DP IV but the pyrrolidines are much better ones. As suggested from our calculations of the dipeptide esters, just such a conformation is favored, where the negatively charged oxygen atoms (and therefore producing a negative molecular electrostatic potential) are placed in the assumed direction of attack. But the pyrrolidines form in this direction a positive molecular electrostatic potential as it occurs in the substrates (see Figs. 1–3). Therefore, an interaction with the enzyme should be much stronger than with the dipeptides possessing a carboxyl group. From all these arguments of our calculations it seems to be justified to conclude, that there is only one probable recognition conformation of substrates which agrees with all experimental findings and computational results. This is the conformation of substrates with the following characteristic dihedral angles: $\Psi_1 = 85°$, $\omega_1 = 180°$, $\Phi_2 = -75°$, $\Psi_2 = 80°$. This structure is shown for Pro-Pro in Fig. 5, middle.

It has to be stated that all of our results should not be influenced by any interactions with a solvent, because on the one hand all conclusions are based on considerations of sterically impossible structures which occur in the gas phase as well as in a solvent phase and on the other hand, the situation in the active site of the enzyme is in principle probably more similar to the gas phase than to the solvent phase.

CONCLUSIONS

We have used the ECEPP83-method to investigate the conformational properties of some substrates and nonsubstrates of DP IV, as well as some dipeptide-ethyl esters which model the acyl enzyme. A stepwise analysis, involving increasingly flexible substrates and including experimental information at all points, led to only one conformation recognizable (and cleavable) by the enzyme. The conclusions were drawn in the following manner:

All conclusions are based on the 10 stable conformations found for Pro-Pro-methylamide (Table 1), the best and most rigid substrate of DP IV.

In comparison with substrates containing alanine in P_1-position we excluded conformations with a cis-peptide bond (conformations 7–10 in Table 1) between the first two amino acid residues because of the high energy of these conformations.

The conformers 1–3 of Table 1 were excluded by comparisons of the different experimental and conformational behavior of the D-Ala-Ala-methylamide and D-Ala-Pro-methylamide. The conformations 1–3 would not explain the experimental findings.

The conformation 4 of Table 1 is the only one which is possible for the nonsubstrate Pro-Pro-Pro-methylamide with respect to the torsional angle Ψ_2. This conformation cannot explain the different experimental behavior between molecules containing proline in P_1'-position and such ones with other amino acids in this position and was therefore excluded from further considerations.

The conformation 6 of Table 1 was excluded to be relevant for the recognition complex and for the cleaving mechanism by consideration of an attack of a serine side chain to the substrates and by comparisons with stable dipeptide ester conformations assuming that the recognition complex, the tetrahedral transition state and the stable intermediate (acyl enzyme) are very similar in all 3 steps without passing high-energy barriers.

The only remaining conformation is characterized by the dihedral angles $\Psi_1 = 82°$, $\omega_1 = 180°$, $\Phi_2 = -75°$ and $\Psi_2 = 79°$ (conformation 5 of Table 1).

Compounds capable of attaining this conformation, are substrates while those incapable of attaining it, are not. Even the slow reaction rate of substrates with D-amino acids in position P_2 and alanine in position P_1 is explainable by a relatively high conformational energy. The theoretical calculations and conclusions allow the assumption that some of the substrate specificities are already formed in the state of the recognition complex. Qualitative conclusions with respect to the strength of inhibitors could also be drawn.

Although neither the X-ray structure of DP IV nor the catalytic mechanism is known, a conformation of substrates of DP IV could be determined which can explain many substrate specificities and allows first indications with respect to a possible catalytic mechanism by dipeptidyl peptidase IV.

From all these computations indications for the synthesis of new inhibitors of DP IV could be given.

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