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A rationally designed oligopeptide shows significant conformational changes upon binding to sulphate ions☆

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Abstract

Oligopeptides that interact with oxoanions were developed by rational design methods. The substrate-binding site of the enzyme purine nucleoside phosphorylase served as a model for the design of the ionophores. The amino acids involved in the complexation of oxoanions were linked through flexible spacer residues. These spacers were chosen such that the relative orientation of the interacting amino acids was conserved. Several peptide sequences were preselected based on intermolecular H-bond frequencies. These frequencies were calculated from molecular dynamics trajectories of the corresponding peptide–anion complexes and used to score the binding properties of the peptides. The most promising peptides were prepared using solid phase peptide synthesis. Anion binding of the peptide ionophores was screened using circular dichroism (CD) and confirmed by NMR spectroscopy. CD measurements performed in methanol revealed a significant conformational change of a linear undecapeptide upon binding to sulphate ions. Two-dimensional-NMR experiments confirmed that a conformation with high helical content is formed in the presence of sulphate ions. These conformational changes induced by the anion stimulate the development of new transduction mechanisms in chemical sensors.

Keywords: Chemical sensors; Ionophores; Molecular modeling; NMR spectroscopy; Oxoanions; Peptides

1. Introduction

Analytical techniques that are on-line, on-site and automated are becoming increasingly important. Chemical sensors are attractive tools for continuous monitoring and controlling automated processes and are therefore ideally suited to meet these future trends (Spichiger, 1998). In the last decade, a remarkable number of such sensors for oxoanions (e.g. phosphate and sulphate) has been described. These include potentiometric electrodes (Carey and Riggan, 1994; Tsagatakis et al., 1994; Antonisse and Reinhoudt, 1998, 1999; Bühlmann et al., 1998; Fibbioli et al., 2000) and enzymatic biosensors (Conrath et al., 1995; Kinoshita et al., 1995; Cosnier et al., 1998). However, most of these devices still hardly meet the requirements for practical applications due to lacking sensitivity, selectivity and lifetime. If these characteristics are to be improved, a special emphasis will have to be laid on the recognition elements used (host compounds, ionophores).

These shortcomings are in sharp contrast to the favourable properties of a large number of proteins that selectively interact with oxoanions (Chakrabarti, 1993). These protein structures usually bind to analyte molecules with a high selectivity, and may therefore serve as models for the host design. Nevertheless, few attempts have been made so far to develop ionophores (e.g. synthetic peptides) that are based on the molecular recognition of ions by enzymes (Schmidtchen and Berger, 1997; Ishida and Inoue, 1999). Natural and artificial peptides that act as ionophores, mainly for cations, are known (Molle et al., 1989; Köck et al.,

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1992; Stefanac and Simon, 1967). The depsipeptide valinomycin is widely used as a highly selective ionophore in potassium-selective electrodes. However, despite their favorable characteristics, there are only few reports on the use of peptide ionophores as recognition elements in chemical sensors (Behm et al., 1985).

A promising approach to develop recognition elements for use in chemical sensors is therefore to use protein structures as models for the recognition of analyte molecules (Giannis, 1993; Luthman and Kacksell, 1996). Our work aimed at designing synthetic oligopeptides based on molecular recognition sites of oxoanions, such as sulphate and orthophosphate, found in enzymes. As a model enzyme, we chose the phosphate-binding protein purin nucleoside phosphorylase (PNP, E.C. 1.2.4.2.), which is involved in the reversible phosphorolysis of nucleotides (Friedkin and Kalckar, 1961; Krenitsky, 1967). From both crystallographic data (Ealick et al., 1990) and binding studies in solution (Kierdaszuk et al., 1997) it is evident that inorganic phosphate as well as sulphate ions are bound by the enzyme. The anion binding site of the enzyme is formed by the residues Ser33, Arg84, His86 and Ser220 which are hydrogen-bonded to the ion. Furthermore, electrostatic interactions via the Arg84 residue contribute to the recognition of the anion. The formation of such ion pairs between the guanidinium group and oxoanions is well-documented in biological systems (Hannon and Anslyn, 1993).

For the design of the peptide ionophore, the residues found in the anion-binding site of the enzyme were linked through flexible spacers. These spacers were chosen such that the residues involved in the complexation of the anion are able to adopt a geometry similar to that found in the enzyme complex. Gly, Leu as well as *n*-aminoalcanoic acids were selected as spacing units for the following reasons: (a) the side-chains of these residues do not interact with ions, and (b) they balance the lipophilicity so that the peptide is soluble in organic solvents. We aimed at providing a sufficient solubility in nonaqueous solvents in order to enhance the reactivity of the ionophores when applied to apolar sensing layers.

2. Experimental

2.1. Molecular modeling

Coordinates of phosphate-binding residues of PNP were extracted from the Brookhaven Protein Data Bank data (pdb code: 1ula) and linked with the spacing amino acids using the Sybyl 6.3 package (Tripos Inc., St.Louis, USA) (Bernstein et al., 1997). The derived peptides were energy-minimized with the Amber 4.1 program using the parm94.dat (all-atom) parameter set (Cornell et al., 1995). In order to yield a picture of the conformational space that is occupied by the designed peptides MD calculations were performed. For each peptide, a total of 50 different initial geometries were derived by performing a 200 ps MD calculation using a simulated annealing procedure and saving the conformation every 2 ps, starting after 50 ps. Finally, these conformations were optimized by performing a 200 ps molecular dynamics run at 300 K. All simulations were carried out for the free peptides as well as their complexes with dihydrogen phosphate. A formal charge of $+1$ was assigned to the guanidino group of the arginine residue. Frequencies of phosphate–peptide hydrogen bonds were determined as described in (Demuth, 1999). Non-standard residues were parameterized according to a previous procedure (Besler et al., 1990). All simulations were performed in vacuo to minimize computational cost although the electrostatic interactions between the anion and the peptide residues are likely to be overestimated.

Flexible docking of the peptide (residues 1–8 only) to the sulphate anion was performed using the recently described Lamarckian Genetic Algorithm (GA) implemented in AutoDock 3.0 (Morris et al., 1998). Firstly, a three-dimensional (3D) grid (size: 22.5 Å $\times$ 22.5 $\times$ 22.5 Å, resolution: 0.375 Å) was centred on the sulphate anion, for which RESP atomic charges had been previously calculated (Bayly et al., 1993). Steric and electrostatic interaction energies between the anion and C,H,N,O probe atoms were pre-computed using default AutoDock settings (Morris et al., 1998). A total of ten independent GA runs were performed always starting from a random orientation of the peptide in the 3D lattice and random torsion angles for the freely rotating bonds (backbone phi, psi angles of Ser1-Gly2; side chains of Ser1, Arg5). Default GA settings (number of generations, number of energy evaluations, mutation and crossover rates, Cauchy distribution, Solis & Wets local search) were used (Morris et al., 1998). The most favoured bound state for each independent docking run was finally saved.

2.2. Peptide synthesis

The peptides were prepared using standard solid-phase peptide synthesis applying *N*-α-(9-fluorenyl)methoxycarbonyl (Fmoc)/tert.Butyl protecting group strategy (Rist et al., 1998). The peptides were purified by preparative RP-HPLC ($>98\%$) and lyophilized prior to use. Correct mass of the peptides was determined using Electrospray-MS (**1**: calc. 884.8 amu, exp. 884.4 amu; **2**: calc. 1109.3 amu, exp. 1108.7 amu; **2a**: calc. 1025.2 amu, exp 1024.7 amu; **5**: calc. 1230.5 amu, exp. 1229.8 amu; peptide sequences see Table 1).

2.3. CD and NMR measurements

CD measurements were recorded on a Jasco 720A Spectropolarimeter at $T = 20.00 \pm 0.02$ °C in nitrogen atmosphere using quartz cuvettes with an optical path length of 0.1 cm. All spectra were corrected for the background of the pure solvents containing the respective anions. The spectra were recorded at wavelengths between 195 and 250 nm with a speed of 20 nm min^{-1}, a sensitivity of 5 mdegrees, and a spectral bandwidth of 1 nm. Each spectrum was repeated three times. The concentration of the peptides was 0.2 mM; the solvent was a mixture of 95% methanol and 5% aqueous Tris(hydroxymethyl)aminomethane (TRIS) buffer (25 mM, pH 7.3).

Samples used for NMR measurements contained 2 mM peptide in 500 μl of a mixture of 95% methanol-d3 and 5% deuterated aqueous TRIS buffer at pH 7.3. Distance constraints were derived from a 200 ms NOESY experiment recorded at 280 K on a Bruker DRX-500 (Kumar et al., 1980; Macura and Ernst, 1980). Assignments for the peptide resonances were performed largely following the sequential resonance assignment protocol developed by Wüthrich (Wüthrich, 1986). Structure calculations by restrained molecular dynamics were done within the DYANA program using the implemented standard simulating annealing protocol (Güntert et al., 1997). Additional dihedral angle constraints for the χ_1 angle were derived from ^3J coupling constants that were measured either directly from the 1D spectra or were extracted by inverse Fourier transformation of in-phase (NOESY) signals (Szyperski et al., 1992). In the final cycles stereospecific assignments could be obtained for all β-protons of residues 4–7. No consistent violations were observed in the final assignment. The resulting 20 lowest-energy structures were superimposed for best fit of the backbone atoms of residues 3–8 and displayed and analysed within MOLMOL (Koradi et al., 1996) (see Figs. 4 and 5).

3. Results and discussion

3.1. Molecular modeling

Molecular modeling studies were carried out to screen a number of peptide sequences (peptides **1** to **12**, see Table 1) that were designed on the basis of amino acid residues involved in the active site of PNP. A preselection was performed based upon calculating the host–guest interaction energies as well as the frequencies of intermolecular H-bonds. A few of these preselected oligopeptides were chosen for synthesis using standard solid-phase techniques. In order to avoid immense synthetic efforts for single positive results, this approach served as a reasonable screening strategy to reduce the number of compounds to be synthesized.

Energy differences between the free peptides and their corresponding complexes with dihydrogen phosphate were calculated according to

$$\Delta\Delta E = \Delta E_{\text{pot,complex}} - \Delta E_{\text{pot,peptide}} - \Delta E_{\text{pot,anion}} \quad (1)$$

Table 1
Energy differences between the free peptides and their corresponding complexes with dihydrogen phosphate, and hydrogen bond frequencies between selected amino acid residues and the phosphate anion (P_i)

Peptide	Sequence[a]	$\Delta\Delta E \pm \sigma$[b] (kcal mol^{-1})	Number of hydrogen bonds[c]			
			$S_1...P_i$	$R...P_i$	$H...P_i$	$S_2...P_i$
1	SGGG**R**G**H**GGGS	-16 ± 13	1.3	2.5	0.0	0.0
2[d]	SGGL**R**L**H**LGLS	-43 ± 12	1.9	2.3	0.0	0.1
3	SXX**R**β**H**XGS	-75 ± 18	1.9	2.2	0.0	0.0
4	*cyclo*-**R**G**H**GGGSGGGGG	-66 ± 16	–	2.3	0.7	0.3
5	*cyclo*-**R**L**H**LGLSLGLGL	-74 ± 14	–	2.4	1.0	0.5
6	*cyclo*-**R**β**H**XGSXXG	-66 ± 10	–	2.9	1.4	0.1
7	*cyclo*-SGGGGGG**R**G**H**GGGGSGGGGG	-75 ± 19	0.2	2.2	0.7	0.3
8	*cyclo*-SLGGLGL**R**L**H**LGGLSLGLGL	-57 ± 19	0.7	2.1	0.2	0.5
9	*cyclo*-SXX**R**β**H**XGSXX	-65 ± 14	0.5	2.7	0.8	0.9
10	**R**G**H**GGGGSGGGGGS	-64 ± 13	0.1	1.3	1.2	0.1
11	**H**GGGGSGGGGGSGGGGGG**R**	-59 ± 15	0.7	1.6	0.3	0.4
12	SGGGGGSGGGGGG**R**G**H**	-55 ± 14	0.1	0.2	0.2	1.2

[a] The amino acids involved in forming the active site of the enzyme PNP, L-serine (S), for L-arginine (R), and L-histidine (L), are highlighted in bold face. As bridging residues the amino acids glycine (G), L-leucine (L), 6-aminohexanoic acid (X) and β-alanine (β) were investigated.

[b] The mean values of $\Delta\Delta E$ and their standard deviations σ were calculated according to Eq. (1) from 50 independent conformations obtained in the molecular dynamic simulations.

[c] The last four columns give the total number of hydrogen bonds between the highlighted amino acids (the side chains O–H or N–H and the backbone amide acting as hydrogen bond donors) and the oxygen atoms of the oxoanion.

[d] **2a** (SGGLAL**H**LGLS) was synthesized to evaluate the role of Arg5 but not used in the calculations.

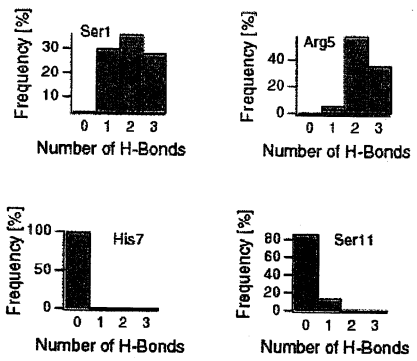

Fig. 1. Hydrogen bond propensities, calculated from simulations for the peptide **2**. The hydrogen bonds were calculated for 50 individual molecular dynamics runs starting from 50 initial conformations. The graphs display the frequency distributions of the total number of hydrogen bonds between Ser1, Arg5, His7 and Ser11 (side chains and backbone amide group) and the oxygen atoms of the oxoanion.

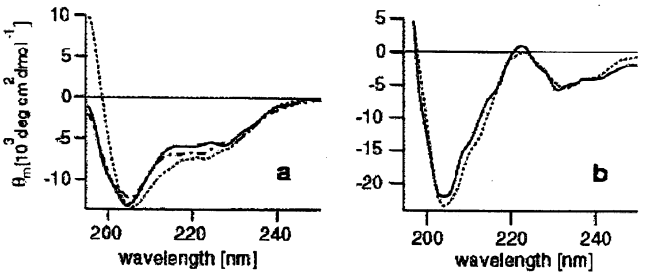

Fig. 2. CD spectra of the peptide **2** (a) and the mutant **2a** (b) (—, free peptide; ....., peptide + 1 equiv. SO_4^{2-}; -.-.-., peptide + 1 equiv. $H_2PO_4^-$).

According to Table 1, the interaction energies of the peptides **1** to **12** in contact with $H_2PO_4^-$ are negative which means a yield of free interaction enthalpy. The values calculated for the peptides **1** to **12** do not differ significantly, with exception of peptide **1**. A series of mutants of the peptides with one serine residue replaced by a second arginine (not shown) displayed significantly increased free interaction energies. It was assumed that in this case a share of free energy is derived from electrostatic interactions to the second arginine residue. However, in reality this gain would be compensated by the increased desolvation energy. Calculations needed to be performed in vacuo to prevent excessive time requirements for the computations. However, ranking of the peptides of similar charge with respect to sulphate binding and structuring should still be possible.

The hydrogen bond occupancies (Table 1) were calculated from molecular dynamics trajectories of the corresponding peptide–phosphate complexes and then used to score the binding properties of the peptides (Demuth, 1999). The calculations showed an average of more than two hydrogen bonds between the arginine guanidinium unit and the phosphate for most of the peptides. Thus, the results confirmed the strong interaction between arginine and oxoanions. They also showed that the only residues Ser1 and Arg5 participated in binding of the oxoanion in contrast to the situation encountered in the enzyme PNP. However these data are in perfect agreement with our observations from the structure elucidation from 2D NMR data and with the results docking experiment. Fig. 1. The propensities of the peptides to form hydrogen bonds were significantly lower for the linear peptides with long chains (peptides **10** to **12**). Hence, the results showed that the entropic loss due to structuring of the peptides upon binding must be minimized through cyclization or by using short chain Length. In view of these results, it was decided to start with the synthesis of the simplest peptides **1**, **2** and **5** in order to investigate the impact of the pre-selection procedure.

3.2. Circular dichroism experiments

Firstly, structuring of the peptides upon binding was studied using circular dichroism (CD) spectroscopy. CD measurements were performed for the peptides **1**, **2** and **5** in the presence of dihydrogen phosphate, sulphate, nitrate, acetate and chloride ions. All experiments were carried out in methanol, since this solvent is frequently used to simulate lipophilic environments encountered in apolar sensing layers.

The CD measurements of peptide **2** (Fig. 2a) showed significant spectral changes when one equivalent of sulphate ions was added to the peptide solution. However, these spectral changes were only weak for the peptides **1** and **5** (not shown). The increase in ellipticity at wavelengths below 200 nm as well as a corresponding decrease in the range from 210 to 220 nm for **2** can be attributed to an increased helicity of this peptide's anion complex. Neither phosphate ions (see Fig. 2a) nor any other of the anions investigated induced a change in the CD spectra similar to sulphate ions. Hence, it was postulated that the conformational changes are not caused by unspecific effects, such as an increase in ionic strength, but rather by a specific intermolecular interaction. This finding was supported by a titration experiment where up to three equivalents of sulphate ions (relative to the peptide) were added in small aliquots (not shown). The titration runs showed that the observed changes in ellipticity significantly depend on the concentration of sulphate ions in the sample.

In a control experiment, further CD spectra were recorded for the peptide **2a**, a mutant of 2, with arginine replaced by alanine. Fig. 2b shows that the mutant **2a** displayed no conformational change upon addition of sulphate. As a conclusion, it is obvious that: (a) the arginine residue plays an important part in the binding of sulphate ions, and (b) unspecific effects do not contribute to the spectral differences observed for

peptide **2**. Since this peptide seemed to be a promising candidate as an ionophore for oxoanions, all further investigations were continued with this molecule.

3.3. NMR experiments

One-dimensional ^{1}H NMR spectra of **2** displayed remarkable changes upon addition of sulphate or phosphate although the changes were much smaller for the latter. Therefore, the further structural characterization was restricted to the sulphate–peptide complex. The chemical shift changes observed upon addition of sulphate to the peptide are included in Table 2. From these data it is obvious that the largest changes occur for residues 2–5. Furthermore, the structuring of the peptide is apparent from changes in the magnitude of the $^3J_{HN\alpha}$ scalar couplings upon binding (Karplus, 1959; Bystrov, 1976). For the complex, these couplings are significantly decreased (see Table 1) towards values typically observed in stable helices in globular proteins (about 4 Hz) (Pardi et al., 1984; Wang and Bax, 1996).

In order to further structurally characterize the sulphate-bound form of **2**, we have applied two-dimensional NMR. Fig. 3 shows a comparison of the NOESY amide proton region between the free and the sulphate-bound form. The presence of strong sequential H^N,H^N NOEs in the latter indicates that the complexed form is ordered. The free form is devoid of structurally well-defined parts (Wüthrich, 1986). The NMR-restrained MD calculations converged to an average RMSD to the mean structure of 0.38 and 1.4 Å for the backbone and side-chain heavy atoms of residues 3–8, respectively. The ensemble of the 20 lowest-energy conformers superimposed for best fit of the backbone

Table 2
Chemical shift deviations $\Delta\delta = \delta_{complex} - \delta_{free}$ of peptide **2** between its sulphate complex and the free form (amide protons, H^N, and H^α protons), and $^3J_{HN\alpha}$ scalar coupling constants of peptide **2**

Residue no.	$\Delta\delta$ (H^N)* (p.p.m.)	$\Delta\delta$ (H^α)* (p.p.m.)	$^3J_{HN\alpha}$ complex (Hz)	$^3J_{HN\alpha}$ free (Hz)
1	–	0.25	–	–
2	0.29	−0.18/−0.01	–	–
3	0.80	0.17/0.08	–	–
4	−0.21	−0.28	4.7	6.8
5	0.80	−0.34	3.9	8.1
6	−0.08	−0.21	3.4	6.2
7	−0.13	−0.18	6.0	7.3
8	−0.30	−0.06	6.9	7.4
9	−0.39	0.01/−0.03	–	–
10	−0.29	0.01	8.4	7.5
11	−0.25	−0.06	8.0	7.1

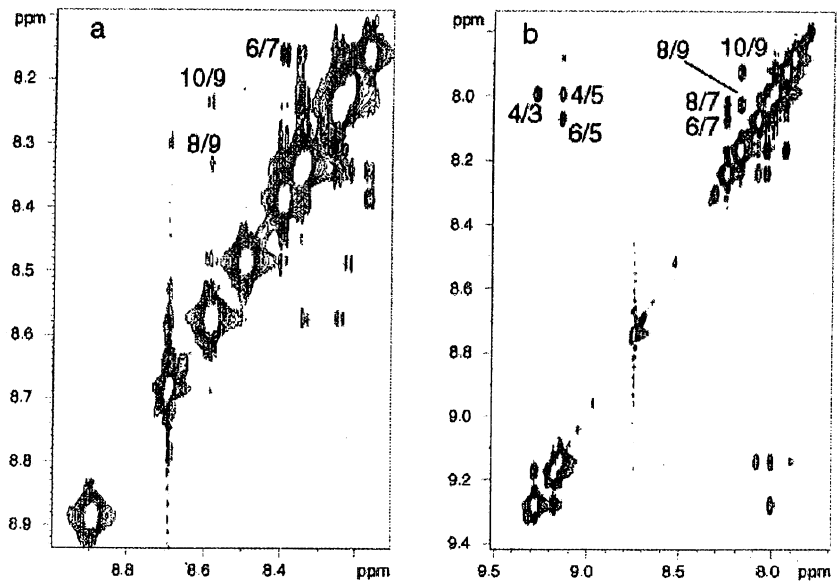

Fig. 3. Expansions of 200ms NOESY spectra of free (a) and SO_4^{2-}-complexed (b) peptide **2**. The annotation of the cross peaks refers to the sequence positions of the amide protons.

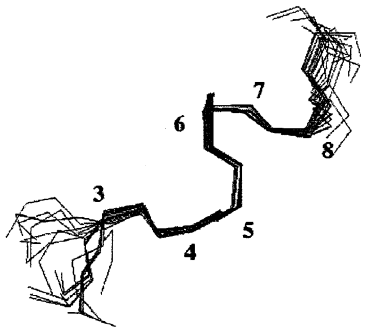

Fig. 4. Representation of the conformations derived from two-dimensional NMR experiments of the sulphate complex of **2**, displaying backbone traces of the superposition of the 20 lowest-energy DYANA structures. The assigned numbers refer to the Cα positions.

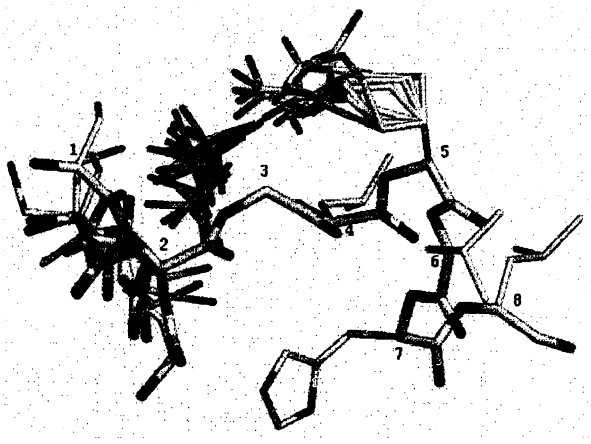

Fig. 5. Ensemble of docked sulphate complexes (peptide **2**) superimposed.

atoms of residues 3–8 is shown in Fig. 4 and displays an α-helical fold of the backbone atoms of residues 3–7.

3.4. Docking

Since proton NMR spectroscopy cannot be used to localize sulphate directly, the peptide (residues 1–8 only) was docked to the sulphate anion using a recently described Lamarckian genetic algorithm (GA) starting from randomly positioned sulphate (Morris et al., 1998). This automated flexible docking procedure suggests a preferred clustering of the anion around the N-terminal half of the peptide (Fig. 5). Salt bridges to the Ser1 N-terminus and(or) to Arg5 side-chain atoms, as well as hydrogen-bonds to Gly2, Gly3, Leu4 backbone nitrogen atoms and(or) Ser1, Arg5 side-chains are common to the ten best orientations found. Interestingly, the proposed docking solutions are in agreement with the above-reported NMR data suggesting that sulphate binding mainly involves backbone atoms of residues 2–5 of the undecapeptide and are supported by the results from the mutant **2a**.

Initiation is an important step for forming helices. N-capping residues forming side-chain hydrogen bonds to the backbone atoms of the first turn significantly help to stabilize the first helix winding (Penel et al., 1999). We do suspect that the sulphate ion is taking over the stabilizing role of these N-capping residues by forming appropriate hydrogen bonds to backbone amide protons of residues 2–4. Backbone carbonyl groups are thus optimally oriented for inducing the first helical turn (Fig. 5). It is remarkable to note that a noncyclic undecapeptide is able to adopt a unique conformation in solution.

4. Conclusions

Using rational design we have been able to develop a peptide that binds sulphate ions and thereby adopts a unique structure. However, for successful application in a chemical sensor the structuring of the peptide needs to be transformed into a measurable signal. It is evident that neither CD nor NMR spectroscopy can be practically used to detect sulphate binding in a commercial chemical sensor. The fact that the recognition of an anion induces a transition into a defined conformation of the host molecule could nevertheless stimulate the development of new transduction mechanisms. As an example, peptide derivatives containing fluorophores at well-defined positions can be synthesized. These derivatives can designed in such a way that the fluorophores come into proximity upon binding of the ion and thereby intramolecularly quench their fluorescence emission through fluorescence resonance transfer. We are now focusing our work on introducing further functionality to the peptides in order to apply the system to the detection of sulphate ions.

The use of peptides turned out to be advantageous in host design: using solid phase peptide chemistry, the syntheses are fast and relatively simple, and parameters, such as the peptides' rigidity and lipophilicity, can be modified more easily. In contrast to many conventional synthetic host molecules, peptides can be tailored specifically and rationally according to a given application. Hence, the use of peptides is very favourable in view of a future application in sensitive layers. Again, the building blocks needed for a covalent attachment of the peptides to a polymer or a surface can be introduced conveniently on a polymer support.

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