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Short communication

Characterization of protein mixtures by ion-exchange chromatography coupled on-line to nuclear magnetic resonance spectroscopy

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Abstract

The first example of HPLC–NMR analysis of proteins is reported. By this means, a mixture of hen egg lysozyme and horse heart cytochrome was separated and identified. The chromatographic separation is based on ion-exchange HPLC, which is likewise for the first time coupled to ^{1}H NMR. In the stop-flow mode, characteristic one-dimensional ^{1}H spectra, two-dimensional total correlation data sets (TOCSY), and nuclear overhauser effect correlation spectra (NOESY) of the two proteins were obtained. In addition, lysozyme from crude hen egg white was unambiguously characterized by using a stop-flow HPLC–NMR-TOCSY experiment. These experiments extend the applicability of HPLC–NMR to the rapid analysis of protein mixtures.

Keywords: Nuclear magnetic resonance spectrometry; Ion-exchange chromatography; Proteins

1. Introduction

Whereas on-line coupling of HPLC with an NMR detector can now be considered an established technique [1,2], there are still relatively few cases where this highly efficient analysis method has really been applied. Publications have focused on the analysis of metabolites in body fluids [3–5] and natural products [6–9], and, to date, only low-molecular-mass analytes have been characterized using reversed-phase chromatography with $^{2}H_2O$ and CH_3CN as eluents. Recently, Lindon et al. extended HPLC–NMR to the analysis of small tripeptide libraries [10]. However, no studies of biological macromolecules have been published yet, although ion-exchange chromatography is widely used in purifications and analysis of protein mixtures [11] and NMR spectroscopy is the method of choice for the characterization of the three-dimensional structure of small proteins in solution with great success in structural biology [12,13]. An on-line coupling of these two techniques offers many potential applications, for example, in profiling crude protein mixtures, or in the analysis of protein–protein or protein–ligand complexes in biological matrices. A HPLC–NMR analytical characterization of such mixtures is thus a relevant challenge. In this communication we present the first application of HPLC–NMR on-line coupling for rapid characteriza-

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tion of protein mixtures. This includes also the first example of coupling ion-exchange chromatography to NMR spectroscopy. We demonstrate that it is possible to get well resolved proton spectra from injection mixtures of a few milligrams of medium size (M_r 14 000) proteins. Under optimal chromatographic conditions, homonuclear two-dimensional NMR experiments (total correlation spectroscopy, TOCSY and nuclear overhauser effect spectroscopy, NOESY) can be performed in stop-flow mode with single proteins, as demonstrated for lysozyme in hen egg white.

2. Experimental

The HPLC–NMR analysis of proteins was performed on a Bruker DMX 600 spectrometer (operating at 600.13 MHz ^{1}H frequency) equipped with an inverse flow probe (capturing 240 μl of the peak volume). The probe was connected to a HPLC system consisting of a Bruker pump, a Rheodyne injection valve and a Bischoff UV spectralphotometer. For chromatography, a cation-exchange Nucleogel SCX column from Macherey & Nagel was used (50×4.6 mm). A gradient was chosen, programmed from 100% buffer A (^2H_2O or H_2O, 0.02 *M* KH_2PO_4, pH 6.0) to 100% buffer B (^2H_2O or H_2O, 0.02 *M* KH_2PO_4, 0.5 *M* NaCl, pH 6.0) over 15 min. The parameters of the NMR experiments, including the pulse sequences, were as previously described [14]. The samples, a mixture of 25 mg hen egg lysozyme and 25 mg horse heart cytochrome *c* (purchased from Sigma) and 100 mg lyophilized hen egg white [11], each dissolved in 1 ml buffer A, were injected without additional sample treatment.

3. Results and discussion

Although operating with solvents containing a large amount of inorganic salts which can effect the NMR acquisition, the use of ion-exchange chromatography is better suited for HPLC–NMR analysis of proteins than a reversed-phase system, since an aqueous eluent composition without any organic modifiers or co-solvents can be applied. This has the advantage that the chromatographic conditions are mild and more physiological and should avoid the denaturation of the proteins during chromatography. A conformational change would also significantly change the detected NMR spectra. Additionally, the relatively inexpensive ^2H_2O-based solvent system provides the possibility to apply simplified mono-solvent suppression techniques as there is no second intense solvent signal. HPLC–NMR stop-flow mode was chosen in this study because of its intrinsic higher sensitivity and spectral resolution compared to on-flow analysis. Two-dimensional correlation NMR experiments, which are crucial for the characterization of proteins, cannot be performed in the on-flow mode. To keep the chromatographic conditions as simple as possible, a cation-exchange column was

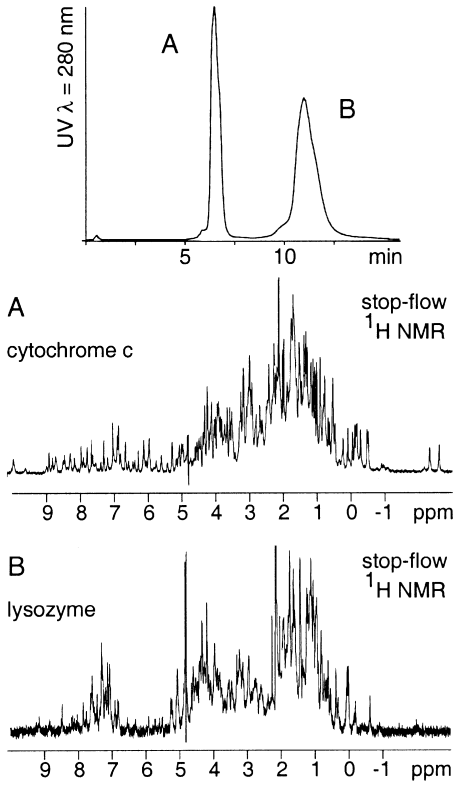

Fig. 1. Cation-exchange UV chromatogram of a mixture of cytochrome *c* and lysozyme and the stop-flow HPLC–NMR spectra of the two peaks. Parameters: buffer solutions prepared with ^2H_2O, nonlinear gradient, injection volume 100 μl, NMR 128 scans, 32 K data points, spectral width 10 KHz, 2 s presaturation of residual HO^2H.

used following the method described by Li-Chan et al. [15], using a phosphate-buffered solvent system applicable to HPLC–NMR [2]. To optimize the chromatographic and in particular the NMR conditions, an equimolar mixture of cytochrome *c* (M_r 13 000, isoelectric point pI = 10.6) and lysozyme (M_r 14 300, pI = 11) was studied first. After injection of 100 μl of the mixture (corresponding to 2.5 mg of each protein) the chromatographic peaks were analyzed in the HPLC–NMR stop-flow mode [2].

Fig. 1 shows a typical chromatogram and the stop-flow ^{1}H NMR spectra obtained for the equimolar mixture of lysozyme and cytochrome *c*. Due to the high ionic strength and changes in salt concentration over the gradient, retuning of the NMR probe was necessary for each peak. As expected, the spectra are crowded with a high amount of overlap and broad linewidths, but the signal quality was acceptable considering the solvent system used. The signal patterns of the two spectra are characteristically different relative to each other. For lysozyme, a two-dimensional (2D) TOCSY [16] experiment was then performed under identical chromatographic conditions to resolve the overlap (Fig. 2A). This two-dimensional experiment establishes connectivities of protons due to isotropic mixing during a spin-lock period and reveals connectivities of scalar coupled protons. The 2D-TOCSY experiment is one of the keys to the assignment of protein residues [12,13] and is therefore essential for the structural analysis of proteins. Due to the use of ^2H_2O, no amide protons are visible (fast exchange with deuterons). In H_2O-based solvent, the amide protons which are important for the assignment of the protein backbone are detectable in the NMR spectra. This is demonstrated by an HPLC–NMR-NOESY experiment which shows the crosspeaks correlating spatially close protons via dipolar coupling (Fig. 2B) [17].

Obviously, high quality two-dimensional NMR fingerprint spectra from mixtures of proteins can be acquired under typical HPLC–NMR conditions in spite of the low total amount of injected substance (2.5 mg is injected on column; due to peak broadening during chromatography, only ca. 60% of this amount is located in the probe, corresponding to a concentration of 0.4 mM), and the fact that high salt concentrations are present, which can affect the spectrum quality. In conventional off-line NMR analysis of proteins in a 5 mm sample tube, which requires the isolated and purified protein, typically 1

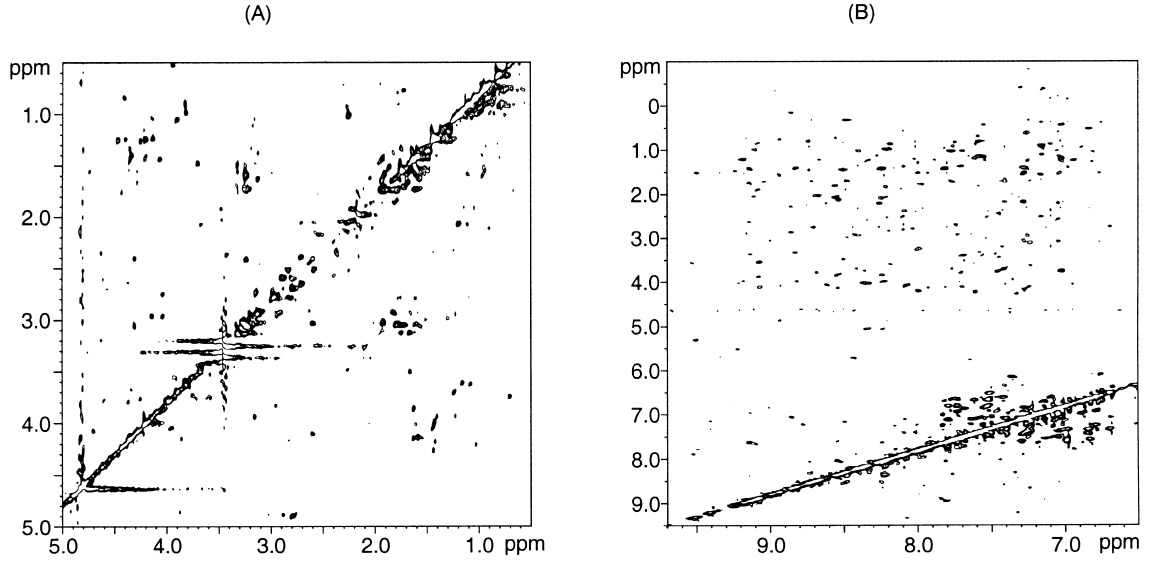

Fig. 2. Selected regions of 2D stop-flow HPLC–NMR experiments performed with the lysozyme peak. (A) Fingerprint region of a TOCSY (chromatography with ^2H_2O), 2.5 mg protein on column, 128 scans, 2 K data points, spectral width 10 kHz, 300 increments, spin-lock 40 ms, resulting in a total acquisition time of 17 h. (B): amide proton region of a NOESY (chromatography with H_2O), 2.5 mg protein on column, 256 scans, 2 K data points, spectral width 10 kHz, 300 increments, mixing time 100 ms resulting in a total experiment time of 21 h.

m*M* concentrations are used. This means for lysozyme, that ca. 8.6 mg have to be dissolved in 0.6 ml solvent. To test the practicability of protein analysis in a real sample, we investigated hen egg white with the described cation-exchange HPLC–NMR coupling technique. 100 mg of lyophilized hen egg white was dissolved in 1 ml ^2H_2O (0.02 *M* KH_2PO_4, pH 6.0) and 500 μl was subjected to HPLC–NMR analysis (Fig. 3).

Careful comparison of the proton spectrum with the reference spectrum (Fig. 1) displayed identity, which was further proven by a TOCSY experiment. The fingerprint region revealed the same crosspeak pattern.

In conclusion, we have demonstrated that HPLC–NMR can be applied to characterize small proteins in mixtures. Homonuclear 2D experiments that are essential for signal assignment and three-dimensional structure elucidation of biomolecules can be performed in the stop-flow mode. Large amounts of structural information can be rapidly obtained because of the short analysis time compared to off-line analysis, where all chromatographic peaks have to be isolated and purified before NMR spectroscopic characterization. This establishes the possibility of the determination of complete structures, including the spatial geometry of small (up to M_r 15 000) proteins, without any purification step prior to HPLC–NMR analysis. A careful validation of the chromatographic procedure is crucial to this method to obtain the highest possible peak separation and therefore good NMR spectra qualities despite high salt concentrations, which are essential for the successful application of ion-exchange chromatography. With the advent of HPLC–NMR at highest fields (750 and 800 MHz) [18,19], the increase in sensitivity and resolution should also permit on-flow experiments and heteronuclear 2D and 3D stop-flow experiments in the future. This work is in progress.

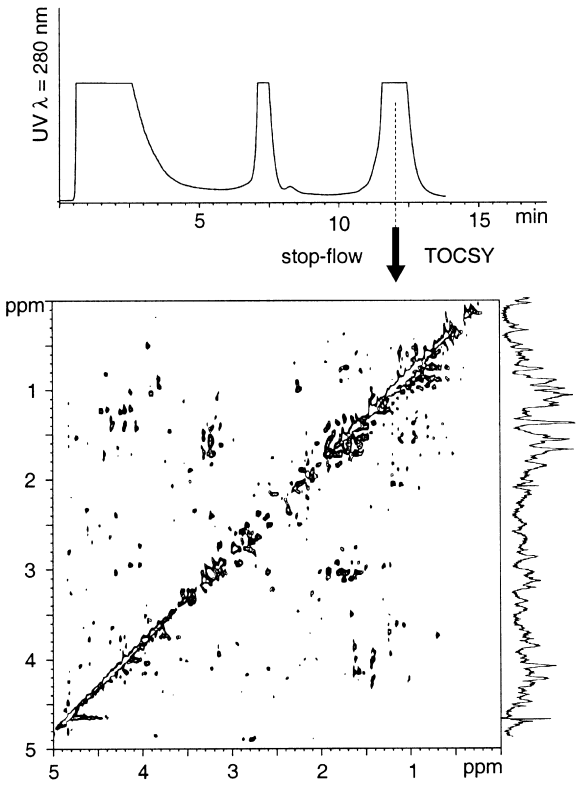

Fig. 3. (Top) UV chromatogram of separation of hen egg white extract, processed under identical chromatographic conditions as described in Fig. 1 (total injected amount 50 mg). The stop-flow NMR spectrum of the lysozyme peak together with a projection of the aliphatic region of a TOCSY experiment is shown at the bottom.

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References

[1] K. Albert, J. Chromatogr. A 703 (1995) 123.
[2] J.C. Lindon, J.K. Nicholson, I.D. Wilson, Prog. NMR Spectrosc. 29 (1996) 1.
[3] M. Spraul, M. Hofmann, P. Dvortsak, J.K. Nicholson, I.D. Wilson, J. Pharm. Biomed. Anal. 10 (1992) 601.
[4] J.C. Lindon, J.K. Nicholson, I.D. Wilson, Adv. Chromatogr. 36 (1996) 315.
[5] M. Spraul, M. Hofmann, P. Dvortsak, J.K. Nicholson, I.D. Wilson, Anal. Chem. 65 (1993) 327.
[6] J. Schlauer, M. Rückert, M. Herderich, B. Wiesen, L. Aké Assi, R.D. Haller, S. Bär, K.U. Fröhlich, G. Bringmann, Arch. Biochem. Biophys. 350 (1998) 87.

[7] S. Johnson, E.D. Morgan, I.D. Wilson, M. Spraul, M. Hofmann, J. Chem. Soc., Perkin Trans 1 (1994) 1499.
[8] K. Albert, G. Schlotterbeck, U. Braumann, H. Händel, M. Spraul, G. Krack, Angew. Chem. 107 (1995) 1102.
[9] K. Albert, G. Schlotterbeck, U. Braumann, H. Händel, M. Spraul, G. Krack, Angew. Chem., Int. Ed. Engl. 34 (1995) 641.
[10] J.C. Lindon, R.D. Farrant, P.N. Sanderson, P.M. Doyle, S. Gough, M. Spraul, M. Hofmann, J.K. Nicholson, Magn. Reson. Chem. 33 (1995) 857.
[11] M.P. Henry, in: R.W.A. Oliver (Ed.), HPLC of Macromolecules, Oxford University Press, Oxford, 1989, p. 91.
[12] K. Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York, 1986.
[13] A. Bax, S. Grzesiek, Acc. Chem. Res. 26 (1993) 131.
[14] G. Bringmann, C. Günther, J. Schlauer, M. Rückert, Anal. Chem. 70 (1998) 2805.
[15] E. Li-Chan, S. Nakai, J. Sim, D.B. Bragg, K.V. Lo, J. Food Chem. 51 (1986) 1032.
[16] D.G. Davies, A. Bax, J. Am. Chem. Soc. 107 (1985) 2820.
[17] J. Jeener, B.H. Meier, P. Bachmann, R.R. Ernst, J. Chem. Phys. 71 (1979) 4546.
[18] U.G. Sidelmann, C. Gavaghan, H.A.J. Carless, M. Spraul, M. Hofmann, J.C. Lindon, I.D. Wilson, J.K. Nicholson, Anal. Chem. 67 (1995) 4441.
[19] U.G. Sidelmann, U. Braumann, M. Hofmann, M. Spraul, J.C. Lindon, J.K. Nicholson, S.H. Hansen, Anal. Chem. 69 (1997) 607.