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Study of conformational effects of recombinant interferon γ adsorbed on a non-porous reversed-phase silica support

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Abstract

Reversed-phase chromatography is a powerful method for separating recombinant interferon γ and one of its analogues differing only by a single amino acid residue. Structural differences of the proteins explain this separation ability as demonstrated from adsorption studies on a non-porous reversed-phase support. To reveal the structural differences occurring in the adsorbed state, two different and independent methods were employed. The variation of the retention with the slope of the linear gradient gave information about the molecular contact area of the protein with the support. For different experimental conditions, these data were correlated with the adsorbent capacities measured on an *n*-octadecyl-modified non-porous silica support. These supports are useful for these types of experiments because the protein is adsorbed exclusively at the external surface of the beads. Moreover, a small amount of protein is necessary to saturate the column, owing to its low capacity.

1. Introduction

Reversed-phase column liquid chromatography (RP-HPLC) is a powerful method for separating and determining peptides and proteins. The separation mechanism is based on the distribution of the amino acid residues (hydrophilic or hydrophobic) on the surface of the molecules.

Chromatographic studies on reversed-phase supports have shown that the retention of proteins is highly dependent on their structure. The chromatographic behaviour of several peptides [1–5] and proteins [6,7] has been examined in the gradient elution mode, by varying the experimental conditions. Moreover, conformational changes may occur during the chromatographic process [8–11]. Fluorescence methods have been used to reveal some structural differences [6,8].

With linear gradient elution conditions, the "linear solvent strength model" [12] was developed to describe the chromatographic parameters related to the physico-chemical properties

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of the solute [13]. Only indirect considerations were used to ascertain the model.

The aim of this work was to study the retention behaviour of two recombinant proteins on a non-porous *n*-octadecyl-bonded silica support in order to elucidate differences in the structural conformation of these proteins and explain the good resolution observed in reversed-phase liquid chromatography.

Janzen et al. [14] and Unger et al. [15] have shown that fast separations of proteins are achieved on non-porous reversed-phase supports. Because of the well defined surface of the adsorbent (the accessible area for adsorption corresponds to the external surface of the spherical beads), we shall show that these supports are useful for fundamental studies relating the molecular contact area of the adsorbed protein to the capacity of the support.

Human interferon γ is a cytokine which plays an important role in the regulation of a variety of immune functions. Its biological activity includes also an antiviral and an antiproliferative action. Recombinant human interferon γ (r-hu IFNγ), derived from *Escherichia coli*, is a basic protein with a relative molecular mass of 17 000. Its sequence contains 144 amino acid residues with no disulphide bridge to stabilize the structural conformation in solution. The recombinant protein is not glycosylated and contains one residue more than the native protein: an N-terminal methionine (position 0). The analogue (A II) that we studied differs from r-hu IFNγ by a single amino acid residue: the asparagine residue in position 25 is deamidated.

Despite this slight difference in the primary structure, the two proteins can be separated by RP-HPLC with a shallow gradient at pH 2. At this pH, the aspartic residue is protonated, so the single amino acid difference cannot explain the separation observed. Probably the good resolution between the two variants is due to conformational variations that are currently being examined by Fourier transform infrared spectrometry [16]. In order to optimize and understand this separation, we studied the retention in the reversed-phase mode of r-hu IFNγ and its variant A II.

2. Experimental

2.1. Apparatus

All chromatographic measurements were performed with a dual-pump system (LC-9A; Shimadzu, Kyoto, Japan) connected to a sample injector valve (Model 7125; Rheodyne, Cotati, CA, USA) equipped with a 20-μl loop. The optical scanning UV detector (Spectra-Physics, San Jose, CA, USA) used was set at 220 and 280 nm. A personal computer (IBM PS2) with Spectra Focus software controls the detector, performs on-line data acquisition via an OS/2 interface and reprocesses peak integrations.

Reversed-phase chromatography was carried out with 40×8 mm I.D. columns packed with non-porous *n*-octadecyl-bonded silica, $d_p = 1.6$ μm, for fundamental studies and with a 250×4.6 mm I.D. Vydac C_{18} column, $d_p = 5$ μm, mean pore diameter 300 Å, in order to separate the proteins in the gradient elution mode.

2.2. Chemicals and reagents

Filtered acetonitrile (HPLC grade) was obtained from Carlo Erba (Milan, Italy) and trifluoroacetic acid (TFA) from SDS (Peypin, France). Water was distilled and deionized in a Milli-Q system (Millipore, Bedford, MA, USA).

Recombinant human interferon γ (r-hu IFNγ) and its analogue (experimental batches) were kindly provided by Roussel-Uclaf (Romainville, France). The r-hu IFNγ was purified from the lysate of genetically modified *E. coli*. The resulting solution of essentially pure r-hu IFNγ (containing a cryoprotector) was lyophilized.

The analogue A II was purified by RP-HPLC using a semi-preparative Vydac C_{18} volume, 218TP (250×10 mm I.D., $d_p = 10$ μm). It was eluted with a gradient of water–acetonitrile containing 0.1% TFA at a flow-rate of 5 ml/min. The pure fractions were lyophilized.

2.3. Chromatographic procedure

Mobile phases were filtered and then degassed with helium.

Linear gradient and isocratic elution were carried out with 0.1% TFA in water (buffer A) and 0.1% TFA in acetonitrile (buffer B) at a flow-rate of 0.6 ml/min.

Capacity measurements were carried out in the isocratic mode by repeated injections of 20 μg of protein until saturation of the non-porous support. The protein was then desorbed using a linear acetonitrile gradient. The amount of protein desorbed was determined from the peak area of the desorbed peak.

Protein solutions were prepared by dissolving the protein in water at a concentration of 0.1 g/l (for the non-porous support experiments) or 1 g/l (for the Vydac C_{18} column experiments). All the experimental data were derived from duplicate measurements, the retention times typically varying by less than 0.5%. The column dead volume was determined from the retention time of sodium nitrate.

3. Results

3.1. Theory

According to the displacement model developed by Geng and Regnier [17], the RP-HPLC retention of a protein expressed as the capacity factor, k', is related to the molar concentration $[D_o]$ of organic solvent in the mobile phase by

$$\log k' = \log I - Z \log[D_o] \quad (1)$$

where I represents the value of k' for $[D_o] = 1.0$ M and Z is the slope of the straight lines obtained when log k' is plotted versus log $[D_o]$.

Kunitani et al. [12] have shown that this relationship for isocratic elution can be extended to the retention of proteins in linear gradient elution:

$$\log \overline{k'} = \log k_0 - S\bar{\psi} \quad (2)$$

where $Z = 2.3S\bar{\psi}$, $\bar{\psi}$ is the value of the volume fraction of the organic solvent as the solute band passes the centre of the column, $\overline{k'}$ is the median capacity factor and k_0 is the extrapolated value of $\overline{k'}$ for $\bar{\psi}$ equals 0.

The retention times of a protein eluted with two gradients that differ in their slope permit the determination of the gradient steepness parameter b. The S and log k_0 values can then be obtained by linear regression analysis based on the values of log $\overline{k'}$ and $\bar{\psi}$:

$$b = t_0 \log \beta / [t_{g1} - (t_{g2}/\beta) + (t_0 + t_d)(t_{G1} - t_{G2})/t_{G2}] \quad (3)$$

$$\bar{\psi} = \psi_0 + \left[t_{g1} - t_0 - t_d - \left(\frac{t_0}{b}\right) \log 2 \right] \Big/ (t_{G1}/\Delta\psi) \quad (4)$$

with $\beta = b_1/b_2 = t_{G2}/t_{G1}$; t_0 is the migration time of the unretained species through the column, t_{gi} is the gradient retention time of the protein corresponding to the gradient time t_{Gi}, t_d is the gradient elapsed time required for the change in solvent B to reach the column inlet and ψ_0 represents the volume fraction of the organic solvent in the eluent at the start of the gradient.

3.2. Gradient elution

In gradient elution, A II is more retained than r-hu IFNγ, as shown in the chromatogram in Fig. 1. The chromatographic behaviour of both variants was carried out on the porous support. The S and log k_0 parameters were determined from the straight line obtained by plotting log $\overline{k'}$ vs. $\bar{\psi}$. A II is eluted after r-hu IFNγ, but its S value is lower than that of r-hu IFNγ. According to the LSS model, S is related to the molecular contact area of the adsorbed protein with the support.

In order to understand better the difference in the S values, a detailed study of the gradient elution behaviour was performed on the non-porous reversed-phase support. The S and log k_0 values were calculated from the plot of log $\overline{k'}$ vs. $\bar{\psi}$ obtained at the same flow-rate, with gradient slopes identical with those in the porous-support experiments (Fig. 2). For comparison, the results are given in Table 1. At 20°C on both columns, the S values obtained for A II are lower than

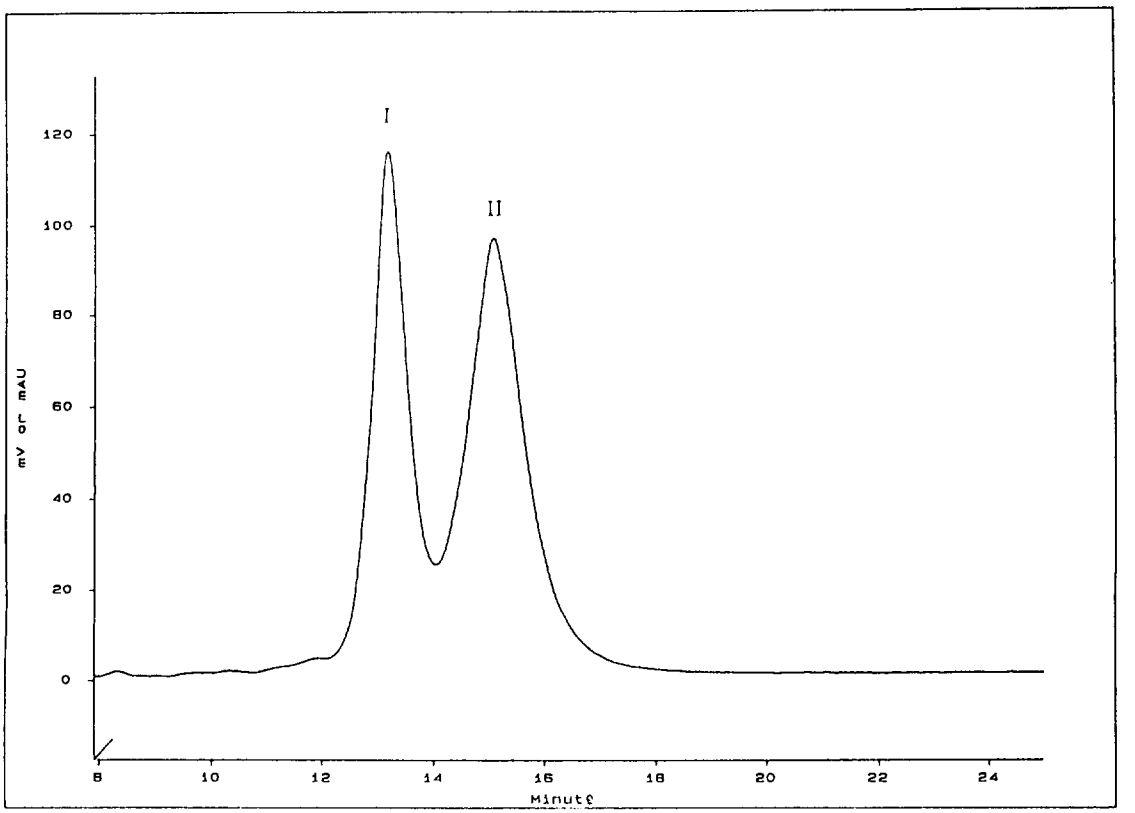

Fig. 1. RP-HPLC of (I) r-hu IFNγ and (II) its analogue AII. Chromatographic conditions: column, 250 × 4.6 mm I.D. Vydac C_{18}; mobile phase, A = 0.1% TFA–water, B = 0.1% TFA–CH_3CN, gradient from 39 to 45% B in 48 min; temperature, 20°C; flow-rate, 0.6 ml/min.

those corresponding to r-hu IFNγ, although the log k_0 values are close.

The influence of temperature on the retention of both recombinant proteins eluted from the non-porous support was studied. The protein retention volume decreases with increasing temperature, but the relative retention of both proteins is almost unchanged, and the S values of A II are lower than those observed with r-hu IFNγ regardless of the temperature.

3.3. Column capacities on the non-porous support

A slight decrease in the column capacity is observed with increasing proportion of acetonitrile in the mobile phase up to 15% (v/v) (Fig. 3). Then the column capacity decreases and there is no retention at acetonitrile contents larger than 35%. This decrease could be explained by a greater surface area occupied by the protein when the solvent content increases. Another effect that could be due to conformational changes is a restricted diffusion process that prevents the protein from adsorption on the whole available surface.

For concentrations of acetonitrile of 0, 10, 15 and 20% in the eluent at 20°C, the column capacity of the A II protein is 1.08, 1.10, 1.05 and 1.07, respectively, times larger than that of r-hu IFNγ. This result shows that the contact surface area of the adsorbed A II molecule is lower than that of r-hu IFNγ.

Fig. 3 shows the variations of the column capacities for r-hu IFNγ with temperature. This study was not carried out with A II because of

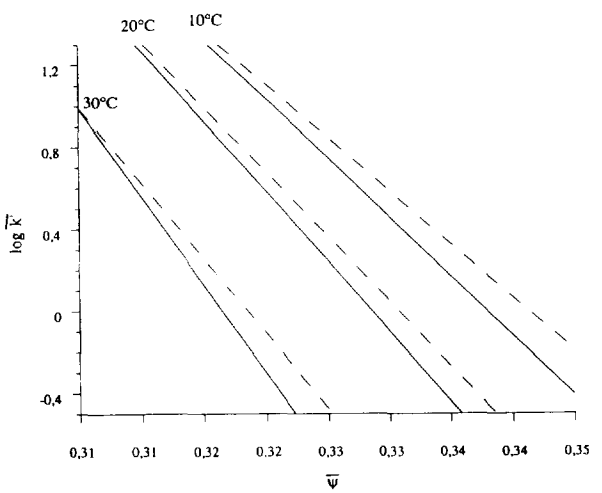

Fig. 2. Plots of log $\bar{k}'$ vs. $\bar{\psi}$ at different temperatures. r-hu IFNγ (solid lines) and AII (dashed lines) eluted on a C_{18} monosphere 40 × 8 mm I.D. column. Mobile phase, A = 0.1% TFA–water, B = 0.1% TFA–CH_3CN, gradient from 30 to 39% B in 18, 36 and 72 min; flow-rate, 0.6 ml/min.

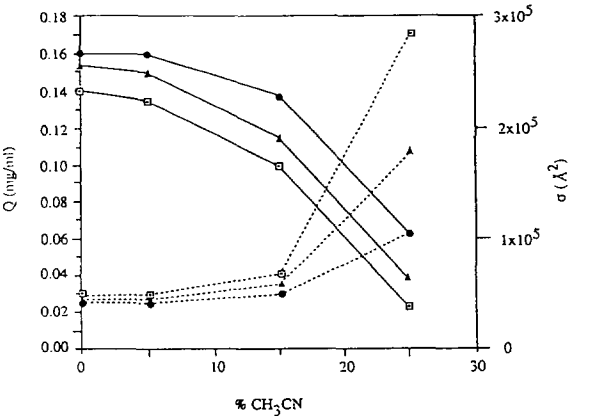

Fig. 3. Plots of the column capacity Q (solid lines) and the surface area σ occupied by r-hu IFNγ (dashed lines) versus the percentage of CH_3CN in the eluent at different temperatures: ● = 10; ▲ = 20; □ = 30°C.

the small amount of protein available. The capacities are lower at higher temperatures, which indicates that the molecular contact area of the adsorbed protein increases with temperature.

4. Discussion

The *n*-octadecyl-modified silica beads are spherical with a diameter of 1.6 μm. The available external surface area per unit volume of column is

$$a_p = \frac{6}{d_p}(1 - \varepsilon) \quad (5)$$

where ε is the interstitial porosity. With $\varepsilon = 0.364$, a_p is calculated to be 2.4 m^2/ml.

The surface area occupied by the protein molecule (σ) can be calculated from the amount adsorbed per unit volume of column. The mean surface area occupied by r-hu IFNγ is 1.07 times larger than that of A II. A similar calculation cannot be applied to the porous support because the adsorbent surface available for protein adsorption is not well defined.

The variations of σ with temperature (Table 2) show that the contact surface area occupied by an adsorbed protein increases with increase in temperature. The surface area occupied is about 5–10% lower at 10°C than at ambient temperature and about 10–20% larger at 30°C. Although small, this variation is significant and more pronounced the higher is the content of organic modifier. This effect can be explained by dehy-

Table 1
Gradient elution parameters (S, log k_0) at different temperatures

Support	Temperature (°C)	r-hu IFNγ		A II	
		Log k_0	S	Log k_0	S
C_{18} porous support	20	16.8	41.4	16.1	39.3
C_{18} non-porous support	10	20.0	58.4	18.0	52.0
	20	22.8	68.4	21.1	62.9
	30	27.7	86.0	23.8	73.4

Table 2
Comparison of the relative surface area $[\sigma/\sigma_{(10°C)}]$ occupied by an adsorbed r-hu IFNγ molecule (isocratic elution) and the relative parameter $S/S_{(10°C)}$ of r-hu IFNγ, determined by gradient elution on the non-porous support

Parameter	Acetonitrile concentration (%)	10°C	20°C	30°C
$\sigma/\sigma_{(10°C)}$	0	1	1.03	1.13
(isocratic elution)	5	1	1.07	1.19
	15	1	1.19	1.38
$S/S_{(10°C)}$		1	1.17	1.47
(gradient elution)				

dration of the protein in solution at higher temperatures that increases its unfolding, and therefore the contact surface area. This phenomenon has already been reported by other workers [2,3,5,6].

It is interesting to compare the results obtained from the capacity measurements with the data obtained in the gradient elution mode. From the variation of the retention volume with the concentration of acetonitrile in the eluent it is possible to determine the parameter S which characterizes the contact surface area (Eq. 2). It has been shown that S is related to the protein molecular mass but varies with the state of unfolding of the adsorbed protein [7]. An empirical relationship was established by Stadalius et al. [13] on reversed-phase supports with acetonitrile–water as mobile phase for peptides:

$$S = 0.48(M_r)^{0.44} \quad (6)$$

According to Eq. 6, the predicted value of S for the proteins studied is 35. This value is underestimated, probably because eq. 6 may not be completely valid for proteins of M_r 17 000.

Experimental S values are higher on the non-porous support than on the porous support. This difference may be explained by a more accessible protein surface in contact with the non-porous support. Further, on the non-porous support, both proteins are eluted with a lower content of organic solvent in the eluent, which results from the much lower capacities of the non-porous support.

The S values increase with increasing temperature, indicating a higher contact area. This affects both variants and consequently the chromatographic separation of the proteins is not improved by changing the temperature.

The ratio of the contact surface area occupied by a molecule, σ(r-hu IFNγ)/σ(A II) = 1.07, is close to the ratio of the S values, S(r-hu IFNγ)/S(A II), as determined from gradient elution retention data. Further, for r-hu IFNγ, the relative variations of σ values with temperature agree closely with those of S values (Table 2). This result validates the gradient elution model and shows that the S parameter is related to the molecular contact area of the protein.

5. Conclusion

This paper has outlined the potential of non-porous supports in the study of the conformational changes of proteins adsorbed on chromatographic supports. They are especially suitable for capacity measurements because of their low and well defined adsorbent surface. Moreover, these types of measurements give information about the adsorption mechanism in reversed-phase chromatography. They validate the model which describes the desorption of the protein as a displacement by solvent molecules. For the first time, a direct relationship between the gradient parameter and the surface area of the adsorbed molecule has been experimentally verified.

Although r-hu IFNγ and its variant differ only by a single residue, the adsorbed surface area of A II is lower than that of r-hu IFNγ, but it is eluted after the latter. This phenomenon indi-

cates that this mutation leads to important conformational changes. Thus, for A II, more hydrophobic exposed groups should interact with the chromatographic support. They are probably located in a region close to the position of the mutant residue.

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