Journal of Chromatography, 371 (1986) 313–333
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 936

MODEL OF PROTEIN CONFORMATION IN THE REVERSED-PHASE SEPARATION OF INTERLEUKIN-2 MUTEINS

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SUMMARY

Thirty muteins* of interleukin-2 were studied by reversed-phase high-performance liquid chromatography in a gradient mode. Values of the stoichiometry-factor Z [from Geng and Regnier, *J. Chromatogr.*, 296 (1984) 15] varied over a 2.5-fold range for these proteins of similar molecular weight and composition. It is proposed that the more hydrophobic and/or more stable proteins have smaller values of Z while the larger Z-values correspond to a higher degree of protein unfolding during reversed-phase retention. The practical utility of this approach was demonstrated when these Z values were used to predict correctly a reversal in elution order for two closely related interleukin-2 muteins, when shallower gradients were used.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is now widely used for the separation of peptide and protein mixtures[1–3]. This has prompted numerous studies aimed at a better understanding of the retention process, for use in improving these practical separations. It has been observed in several RP-HPLC studies that the combination of a hydrophobic column packing plus mobile phases rich in organic solvent leads to the denaturation of protein solutes[4–7]. Many workers believe that protein solutes generally unfold to some extent upon contact with the stationary phase, even from non-denaturing mobile phases[7–10]. That is, the RP-HPLC process is inherently denaturing, in that it drives the protein molecule to unfold so as to expose its more-hydrophobic interior surface for better hydrophobic interaction with the stationary phase. In several cases it has been observed that the RP-HPLC

* The term "mutein" describes a genetically engineered protein expressed from a nucleic acid sequence which has been altered using techniques such as site-specific mutagenesis. Such genetic alterations are designed to result in one or more substitutions, additions, or deletions to the amino acid sequence of the parent protein.

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separation of a single protein yields two peaks[5,8,11,12]: one arising from the native (active) protein, and the other corresponding to denatured protein. These two-peak separations are generally observed for proteins that are less prone to denaturation under the usual conditions of reversed-phase protein separation: low pH and high concentrations of organic solvents in the mobile phase. This suggests for the case of less stable proteins (where generally one band per protein is observed) that the solute molecule is completely denatured upon contact with the column packing.

Geng and Regnier[13,14] have determined the stoichiometry, Z, of the protein-solute–stationary phase interaction, and have shown that the contact surface area of the protein (requiring an equal area on the column packing surface) is proportional to protein molecular weight. This implies a complete unfolding with solute–sorbent contact for all parts of the unfolded protein chain upon reversed-phase sorption. In contrast, retention of a three-dimensional protein structure should show the contact surface area varying with some fractional power of protein molecular weight. For example, based on considerations of geometry, the contact area for a spherical, conformation of a protein should be proportional to the one-third power of molecular weight. However these results are for a mobile phase (formic acid–propanol–water) that is highly denaturing. Results from other studies suggest that considerable tertiary structure can be retained in proteins sorbed onto the reversed phase: (i) more than two peaks are observed for a single protein, implying different degrees of unfolding of the retained molecule[9,15]; (ii) retention cannot be predicted from the group-increments of individual amino acids in the protein[16]; (iii) for many RP-HPLC systems, stoichiometry values, Z, are not proportional to protein molecular weight, MW, as proposed by Geng and Regnier[13], but instead show a dependence on MW^n, where n is less than unity (see refs. 17, 18 and following discussion).

We propose that the stoichiometry factor, Z, for the RP-HPLC retention of a given protein can provide information on the conformation of the retained solute molecule in the stationary phase; *i.e.*, its degree of unfolding or relative denaturation*. Previous studies of the stoichiometry factor, Z, have involved either peptides and proteins of randomly varied structure[13,17,18]. Since Z values vary with protein molecular weight, conformation, and/or other factors, it is not easy to draw general conclusions from these latter studies concerning the role of protein structure in affecting relative unfolding during sorption onto the column packings. In this paper we will present stoichiometry data for a closely related set of muteins of interleukin-2 (IL-2) of *ca.* 15 kD, where amino acids at positions 1, 58, 104, 105 and/or 125 in the parent mutein are substituted by other amino acids. Molecular weight and composition are quite similar for this group of compounds, yet Z values (and presumably conformation) vary markedly. This fact allows us to gain added insight into the conformation of various proteins during RP-HPLC separation.

THEORY

The displacement model of RP-HPLC retention as developed by Geng and

* It has already been shown for hydrophobic interaction chromatography (HIC) that values of Z can be used to infer protein conformation[19,20]. Under conditions where protein denaturation was observed, larger values of Z were also found.

Regnier[13] can be simplified as follows:

$$P_m + Z\,M_a \rightarrow P_a + Z\,M_m \quad (1)$$

Here, a protein molecule P in the mobile phase (m) displaces Z molecules of the adsorbed (a) solvent M that initially cover the surface of the column packing. The retention of the protein molecule, k' (capacity factor), can then be related to the molar concentration, $[D_0]$, of organic solvent in the mobile phase.

$$\begin{aligned}\log k' &= \log I + Z \log(1/[D_0]) \\ &= \log I - Z \log[D_0] \quad (2)\end{aligned}$$

I represents the value of k' for $[D_0]$ equals 1.0 M. When retention ($\log k'$) is plotted against $\log[D_0]$ for isocratic RP-HPLC separation, straight-line curves are obtained whose slope is equal to $-Z$. An alternative approach to obtaining stoichiometry factors for the RP-HPLC process is to use gradient elution, as reviewed in refs. 17, 18 and 21. In this case isocratic retention, k', is assumed to be related to the volume fraction, φ, of organic solvent in the mobile phase as

$$\log k' = \log k_w - S\,\varphi \quad (3)$$

Here, k_w refers to the value of k' for water as mobile phase ($\varphi = 0$), and S is a constant that depends upon the protein solute and the organic solvent (acetonitrile, propanol, etc.) in the mobile phase. Eqn. 3 is generally a good approximation to actual isocratic plots of $\log k'$ *vs.* φ for conditions that are commonly encountered in gradient elution ($1 < k' < 10$). If a particular solute (protein) is separated in two different gradient elution runs, where only gradient time is varied, it is possible to derive values of k_w and S for the protein in that system[18]. That is, isocratic retention parameters as in eqn. 3 can be obtained from two (or more) gradient runs. This is generally more convenient than the isocratic mapping of retention to obtain k' as a function of φ. Two or more gradient runs can also be used to obtain corresponding values of k' ($\bar{k}$) and φ ($\bar{\varphi}$) for each run (see Discussion of refs. 17 and 21).

The value S can be regarded as a stoichiometry factor, similar to the quantity Z. We can derive a relationship between S and Z as follows. We assume that the change in volume upon mixing water with organic solvent to form the final mobile phase is negligible. If the molar volume of the organic solvent is V_m, then

$$[D_0] = 1000\,\varphi/V_m \quad (4)$$

We have from eqns. 2 and 3

$$Z = -\mathrm{d}(\log k')/\mathrm{d}(\log[D_0]) \quad (5)$$

and

$$S = -\mathrm{d}(\log k')/\mathrm{d}\varphi \quad (6)$$

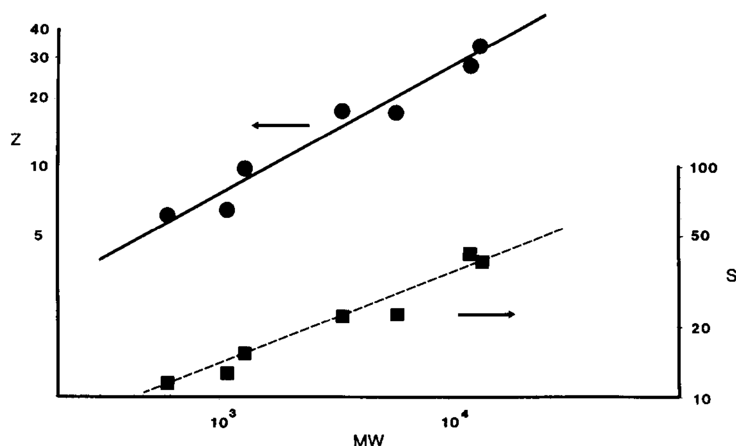


Fig. 1. Comparison of values of Z and S for seven peptides separated in an acetonitrile–water (0.1% TFA) gradient (data from ref. 16). Solutes are: Leucine enkephalin, bradykinin, angiotensin, glucagon, insulin, ribonuclease A, lysozyme. ● = Z values; ■ = S values.

Combination of eqns. 4–6 gives

$$\begin{aligned} Z &= \{d\varphi/d(\log[D_0])\}S \\ &= (d\varphi/d[D_0])\,\{d[D_0]/d(\log[D_0])\}S \\ &= (V_m/1000)\,(2300/V_m)\varphi S \\ &= 2.3\ \varphi\ S \end{aligned} \tag{7}$$

A comparison of values of Z, calculated by eqn. 7 from previously reported S values for a series of peptides (600 < MW < 14 000), is shown in Fig. 1[17]. Both Z and S are seen to vary with molecular weight (MW) as a fractional power ($MW^{0.39}$ for Z and $MW^{0.55}$ for S). This differs from the first-power dependence of Z on MW, reported earlier[13], and is discussed later. Eqn. 2 assumes that the surface of the column packing is completely covered by organic solvent molecules; this is the case only for values of $[D_0]$ above some critical values[14].

In this study we have determined values of Z for correlation with protein structure and conformation. Values of S were determined first from two or more gradient runs (as in ref. 21). Values of Z were then calculated from eqn. 7, using values of $\bar{\varphi}$ calculated as in ref. 21.

$$\bar{\varphi} = \varphi_0 + [t_g - t_0 - t_D - 0.3(t_G/\Delta\varphi S)]\,(\Delta\varphi/t_G) \tag{8}$$

EXPERIMENTAL

Apparatus and materials

The apparatus, column and solvents used were identical to system 2 described previously[22]. The gradients were linear in acetonitrile–water, both solvents containing 0.1% trifluoroacetic acid (TFA).

Values of the column dead time t_0 (1.7 min at 2.0 ml/min) and the dwell time

of the LC system t_D (3.1 min at 2.0 ml/min) were determined as described in ref. 23 and 24. The sources of acetonitrile, TFA, and IL-2 muteins were described previously[22]. The 4,4'-dimethoxybenzophenone was obtained from Fluka (Hauppauge, NY, U.S.A.).

Preliminary runs to confirm accuracy of gradient-derived isocratic retention parameters

The use of gradient elution as in refs. 17, 18, 21 to derive values of the isocratic retention parameters k_w and S requires properly functioning equipment and attention to detail. We therefore carried out initial studies to (i) confirm that valid data were being obtained and (ii) to assess the accuracy and precision of the data.

The chromatographic system has a dwell volume of 6.2 ml, from which t_D is given as $6.2/F$ (F is mobile phase flow-rate in ml/min). The column dead time t_0 was

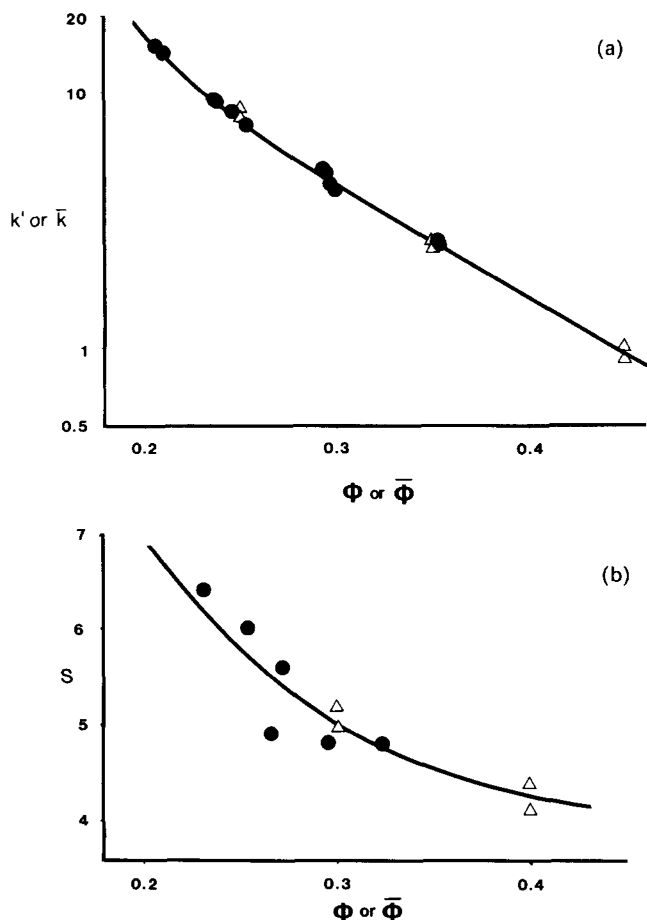


Fig. 2. Comparison of gradient-derived values of $\bar{k}$ and S with corresponding isocratic (direct) measurements for the small molecule 4,4'-dimethoxybenzophenone. Conditions as in Experimental section except 10–50% acetonitrile–water (0.1% TFA) gradient. The procedure for calculation of k', $\bar{k}$, S, φ *and* $\bar{\varphi}$ are given in ref. 18. (a) Values of $\bar{k}$ (or k') *vs.* $\bar{\varphi}$ (or φ); (b) Values of S *vs.* $\bar{\varphi}$. ● = Gradient elution; △ = isocratic elution.

$3.4/F$. Fig. 2 shows experimental isocratic and gradient retention data for the small molecule, 4,4'-dimethoxybenzophenone. Column, eluent and flow were similar to those used later for IL-2 muteins. Values of log k' are plotted against φ in Fig. 2a, while values of S are plotted against φ in Fig. 2b. In each case data from isocratic runs (open triangles) are in close agreement with data from gradient runs (closed circles). This correlation confirms that our equipment and procedures were capable of providing accurate retention data (values of k' and S *vs.* φ) based on gradient runs (see similar comparisons in refs. 21).

We next repeated the study in Fig. 2 with desAla^1(SH^{58}, SH^{105}) Ser^{125} IL-2 and desAla^1(S^{58}–S^{105}) Ser^{125} IL-2. These data are summarized in Fig. 3. Again, there is close agreement between isocratic and gradient measurements, and the gradient plots from several runs (varying F and gradient time t_G) show close adherence to eqn. 3. At this point, we were satisfied that our experimental approach was yielding accurate values of S and k' *vs.* $\bar{\varphi}$.

Precision and accuracy of final data

At least three gradient runs were used for each compound, with gradient times of 20, 40, and 80 min. Values of the isocratic parameters S and log k_w were then obtained for each pair of runs: 20/40, 40/80 and 20/80. Values of S were next compared for the 20/40 *vs.* 40/80 runs, in order to assess the precision of these measurements. For 30 compounds handled in this way, the average error in S (1 S.D.) was $\pm$ 1.8 units, and the average value of S was 24, for an average relative standard deviation (R.S.D.) of 7%. This compares favorably with the precision of gradient-derived values of S in other studies[21]: R.S.D. = 10% for alkyl phthalates as solutes, and $\pm$ 14% for a 50 000-D polystyrene. Other measures of the reproducibility of S values were obtained from 20/80 min pairs for five duplicate runs (R.S.D. = 5%) and from replicate runs for four proteins in a control sample (R.S.D. = 7%, n = 14). On the basis of these comparisons, the precision of reported S values is believed to be about $\pm$ 6%.

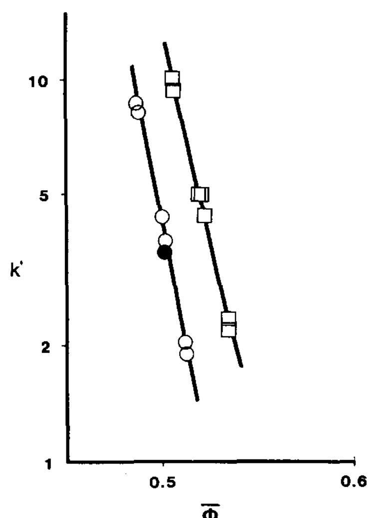
Values of log k' for $\varphi = 0.55$ were also determined from these gradient-derived parameters. For the same five duplicate runs and control-sample replicates cited above, the S.D. for values of log k' (55%) was determined to be $\pm$ 0.08 units.

RESULTS AND DISCUSSION

Summary of mutein structures and properties

The chemistry and structure of the muteins are described in more detail by Kunitani *et al.*[22] (*cf.* references therein). The IL-2 molecule is shown diagramatically in Fig. 4. IL-2 muteins contain substitutions at positions 1, 58, 104, 105 and 125 in the molecule. In the parent protein there are three cysteine groups at positions 58, 105 and 125. Oxidation of two of these thiol groups preferentially leads to a (S^{58}–S^{105}) disulfide bridge. In some of the IL-2 muteins studied here, one of the cysteines is replaced by serine. When this substitution occurs at position 58 or 105, the "natural" disulfide bridge is blocked, and an "unnatural" disulfide (S^{58}–S^{125} or S^{105}–S^{125}) results upon oxidation of the molecule.

There are four methionine groups in the natural molecule, and under mildly oxidizing conditions the methionine at position 104 forms the methionine sulfoxide[22].



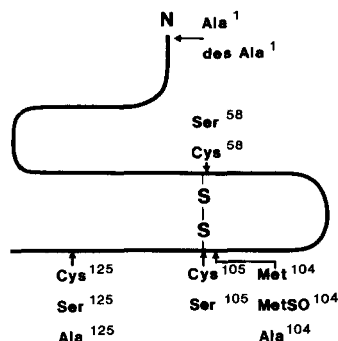


Fig. 3. Comparison of gradient-derived values of $\bar{k}$ and S with corresponding isocratic (direct) measurements for desAla^1(S^{58}–S^{105})Ser^{125} IL-2 and desAla^1(SH^{58}, SH^{105})Ser^{125} IL-2. Conditions as in Experimental section. ○ = Gradient data for cystine-containing desAla^1(S^{58}–S^{105})Ser^{125} IL-2; □ = gradient data for cysteine-containing IL-2; ● = isocratic data for cystine-containing IL-2.

Fig. 4. A simplified schematic diagram of cystine-containing IL-2, showing the positions of the various mutein substitutions.

Another characteristic of IL-2 is its extreme hydrophobicity. In aqueous solutions, it dissolves only with difficulty at pH extremes or with the use of detergents; IL-2 has a relatively high content of non-polar amino acid groups. We will see important consequences of this hydrophobicity in the following discussion.

Dependence of values of S and Z on solute structure

We have commented on the expected correlation of values of S or Z with solute molecular weight. MW; Fig. 1 illustrates this correlation for a particular group of peptides, separated earlier by RP-HPLC[17]. Geng and Regnier[13] found Z to be proportional to MW, in a highly denaturing mobile phase. Stadalius *et al.*[17] summarized values of S *vs.* MW for several studies from the literature (all with acetonitrile–water as mobile phase); a half-power correlation of S (and Z) with MW was observed over the range $600 < \mathrm{MW} < 80\,000$. We believe that this reflects partial folding of typical proteins when they are sorbed onto the column packing. For the RP-HPLC separation of polystyrenes of varying MW[21,25], S (and Z) increases with $\mathrm{MW}^{0.5}$ ($200 < \mathrm{MW} < 233\,000$). From these and other studies[14] it appears that S and Z are determined mainly by solute molecular weight.

When a large group of peptides or proteins are examined it is found that the correlation of S with MW shows significant scatter, suggesting that other factors contribute to the value of S. Specifically Aguilar *et al.*[18] have reported S values for 29 peptides with $400 < \mathrm{MW} < 3300$ (acetonitrile–water gradients) and found a rather poor S *vs.* MW correlation ($r = 0.59$). This can be attributed in part to the small range in MW covered (compared to that in ref. 17) but it also reflects the importance of factors other than MW as contributors to S.

Data for interleukin-2 muteins

Gradient retention data were collected for the present series of compounds (summarized in Table I). Values of S and log k_w were determined from these data (Table II), along with values of log k' for an acetonitrile–water (55:45) mobile phase, log k' (55%). Note that the following discussion is based on S or Z values calculated from the 20/80-min run pairs; these are best values for these and other parameters (see discussion in ref. 21)*.

The compounds in Tables I and II labeled peak A correspond to the methionine sulfoxide that results from oxidation of the methionine-104 residue in the corresponding peak B[22]. We have found that values of S for the peaks A are consistently larger than for the peaks B, by + 6% (± 3%) for the complete set of compounds in Table II. The variation in this S value difference (3%) is smaller than the experimental error (± 6%) of individual values of S; thus there is little value in further analyzing individual values of S for peaks A *vs.* B. In fact, we can improve the precision of a reported S value for the peak B by averaging the peak A value (divided by 1.06) with the peak B value. This was done in the further analysis of the data in Table II (this results in only minor changes in individual S values for the various peaks B). In addition, we have reduced the number of data for further interpretation by one half.

The log k' (55%) values of Table II show a similar correlation for peaks A and B. The value of log k' (55%) for peak A minus peak B is −0.31 ± 0.07 (1 S.D.). The variability in this difference measurement is similar to the experimental error (± 0.08 units) in individual values of log k' (55%). We will therefore not attempt to interpret individual values of log k' (55%) for peaks A *vs.* B, and will average the peak A (+ 0.31 units) and peak B as in the case of the corresponding S values, to obtain best final values of log k' (55%) for the peaks B. Table III summarizes these values of S and log k' (55%) for all compounds studied.

Changes in Z and solute hydrophobicity

Globular proteins are believed to fold in such a way that the hydrophobic residues are tucked inside, with the hydrophilic residues largely on the outside of the molecule. As the protein becomes more hydrophobic, there is an increased driving force toward folding of the protein (in solution) so as to isolate hydrophobic groups from contact with the external aqueous medium, and to minimize the surface area of the folded protein. This suggests that more hydrophobic proteins will be more structured, or at least will resist unfolding to a greater extent in solution. This enhanced conformational stability for very hydrophobic proteins could then persist for the retained molecule, leading to smaller contact areas and smaller values of Z. The Z values of Table III are seen to vary over a wide range: $19 < Z < 49$. This was initially somewhat surprising for a series of proteins of virtually identical molecular

* As discussed in ref. 21, the accuracy of values of S, derived from two gradient runs can be quite dependent on the choice of experimental conditions: the organic solvent concentration at the beginning of the gradient, the gradient time, flow-rate and column volume, and the ratio, β, of the two gradient times used in determining S. Larger values of β generally result in more precise values of S. This favors our use of the 20/80-min data sets for final values of S. Other experimental conditions were also selected for maximum accuracy in derived values of S and Z.

TABLE I

GRADIENT RETENTION DATA FOR INTERLEUKIN-2 MUTEINS

35–60% Acetonitrile–water gradient, 2 ml/min, 30°C. Other conditions as in Experimental section.

Compound	*Oxidation**	*Retention time*		
		*20***	*40***	*80***
$Ala^{1}Cys^{125}$ (Parent)	A-ox	20.02	33.18	57.43
	B-ox	21.04	35.03	61.06
	A-red	22.18	36.30	62.50
	B-red	23.56	38.99	67.78
$Ala^{1}Ser^{125}$	A-ox	18.08	29.60	50.87
	B-ox	18.98	31.41	53.90
	A-red	19.59	31.94	54.75
	B-red	20.98	34.61	59.74
$desAla^{1}Ala^{125}$	A-ox	20.85	34.58	59.48
	B-ox	21.93	36.60	63.13
	A-red	23.02	38.10	66.02
	B-red	24.48	40.92	71.14
$desAla^{1}Ala^{104}Ser^{125}$	B-ox	18.31	30.02	50.68
	B-red	19.99	33.14	56.25
$desAla^{1}Cys^{125}$	A-ox	19.91	33.00	56.14
	B-ox	20.96	34.89	59.83
	A-red	21.84	36.06	62.36
	B-red	23.27	38.76	67.44
$desAla^{1}Ser^{58}$	B-ox	12.94	19.88	32.14
	A-red	21.89	36.41	63.21
	B-red	23.13	38.94	68.01
$desAla^{1}Ser^{105}$	B-ox	13.11	20.72	34.14
	A-red	21.44	35.22	60.49
	B-red	22.82	37.99	66.03
$desAla^{1}Ser^{125}$	A-ox	18.25	29.42	50.45
	B-ox	19.14	31.40	53.76
	A-red	20.07	32.02	54.60
	B-red	21.42	34.48	59.42
$desAla^{1}Ser^{58,105,125}$	A-red	20.57	33.71	57.92
	B-red	21.06	34.44	59.17
$desAla^{1}Ser^{125}$ (control)	A-ox	18.31	29.90	51.12
		18.27	29.79	
		18.25	30.03	
	B-ox	19.20	31.74	54.29
		19.16	31.40	
		19.14	32.96	

* "Oxidation" refers to disulfide oxidation state; "ox" refers to compound containing a cystine (S–S) disulfide bridge, "red" to cysteine-containing (SH,SH) form; "A" refers to compound with Met-104 oxidized to sulfoxide (peak A), "B" to compound with no methionine sulfoxides.

** Gradient time, t_G, in min.

TABLE II

SUMMARY OF RESULTS FOR INTERLEUKIN-2 GRADIENT RUNS

Experimental conditions as in Table I, unless noted otherwise; t_0 = 1.7 min, t_D = 3.1 min. Calculated from retention data of Table I using procedures of ref. 21.

Compound	*Oxidation**	t_G1**	t_G2**	*S*	*log* k_w	*log k' (55%)*
Ala[1]Cys[125] (Parent)	A-ox	20	40	23.4	12.6	
		40	80	23.3	12.5	
		20	80	23.4	12.6	−0.29
	B-ox	20	40	21.4	11.8	
		40	80	22.9	12.6	
		20	80	22.1	12.2	0.03
	A-red	20	40	14.8	8.5	
		40	80	18.2	10.3	
		20	80	16.3	9.3	0.38
	B-red	20	40	14.5	8.6	
		40	80	17.8	10.4	
		20	80	16.0	9.4	0.66
Ala[1]Ser[125]	A-ox	20	40	27.4	14.0	
		40	80	27.4	14.0	
		20	80	27.3	14.0	−1.05
	B-ox	20	40	27.5	14.4	
		40	80	23.4	12.3	
		20	80	25.3	13.2	−0.67
	A-red	20	40	19.6	10.6	
		40	80	22.2	11.8	
		20	80	20.9	11.2	−0.33
	B-red	20	40	18.9	10.5	
		40	80	20.6	11.3	
		20	80	19.7	10.9	0.06
desAla[1]Ala[125]	A-ox	20	40	20.8	11.4	
		40	80	19.7	10.9	
		20	80	20.9	11.1	0.02
	B-ox	20	40	19.6	11.1	
		40	80	18.3	10.4	
		20	80	18.9	10.7	0.30
	A-red	20	40	15.3	9.0	
		40	80	17.9	10.3	
		20	80	16.5	9.6	0.55
	B-red	20	40	14.9	9.0	
		40	80	16.3	9.8	
		20	80	15.6	9.4	0.83
desAla[1]Ala[104]Ser[125]	B-ox	20	40	26.8	13.8	
		40	80	21.1	11.0	
		20	80	23.6	12.2	−0.80
	B-red	20	40	19.7	10.7	
		40	80	25.3	13.5	
		20	80	22.5	12.1	−0.30

TABLE II (*continued*)

Compound	*Oxidation**	t_G1**	t_G2**	*S*	*log* k_w	*log k' (55%)*
desAla[1]Cys[125]	A-ox	20	40	23.8	12.8	
		40	80	29.0	10.3	
		20	80	21.2	11.4	−0.25
	B-ox	20	40	21.6	11.9	
		40	80	18.7	10.4	
		20	80	20.0	11.1	0.05
	A-red	20	40	17.1	9.7	
		40	80	19.4	10.9	
		20	80	18.2	10.3	0.29
	B-red	20	40	16.2	9.5	
		40	80	18.2	10.6	
		20	80	17.1	10.0	0.61
desAla[1]Ser[58]	B-ox	20	40	40.1	17.8	
		40	80	34.1	15.2	
		20	80	36.9	16.4	−3.9
	A-red	20	40	18.7	10.6	
		40	80	20.0	11.3	
		20	80	19.4	10.9	0.29
	B-red	20	40	19.1	11.1	
		40	80	19.0	11.0	
		20	80	19.1	11.1	0.59
desAla[1]Ser[105]	B-ox	20	40	***	***	
		40	80	44.4	19.8	
		20	80	47.8	21.3	−5.0
	A-red	20	40	16.8	9.5	
		40	80	18.7	10.4	
		20	80	17.7	9.9	0.2
	B-red	20	40	16.9	9.8	
		40	80	18.7	10.8	
		20	80	17.8	10.3	0.51
desAla[1]Ser[125]	A-ox	20	40	21.1	10.9	
		40	80	26.8	13.7	
		20	80	23.6	12.2	−0.82
	B-ox	20	40	23.2	12.2	
		40	80	22.7	12.0	
		20	80	22.9	12.1	−0.53
	A-red	20	40	14.6	8.1	
		40	80	20.7	11.1	
		20	80	17.2	9.4	−0.08
	B-red	20	40	13.5	7.7	
		40	80	20.3	11.2	
		20	80	16.2	9.2	0.23
desAla[1]Ser[58,105,125]	A-red	20	40	18.3	10.1	
		40	80	20.5	11.2	
		20	80	19.3	10.6	−0.03

(*continued on p. 324*)

TABLE II (*continued*)

Compound	*Oxidation**	t_G1**	t_G2**	*S*	*log* k_w	*log k' (55%)*
	B-red	20	40	16.7	9.3	
		40	80	19.6	10.8	
		20	80	18.0	10.0	0.11
desAla[1]Ser[125] Control§	A-ox	20	40	25.1	12.9	
		40	80	24.8	12.8	
		20	80	25.0	12.9	−0.86
Control§	B-ox	20	40	23.4	12.3	
		40	80	24.1	12.7	
		20	80	23.8	12.5	−0.54
Control§	A-ox	20	40	24.7	12.7	−0.86
	B-ox	20	40	22.7	12.0	−0.51
Control§	A-ox	20	40	28.8	14.8	−1.07
	B-ox	20	40	27.8	14.6	−0.71

* Oxidation indicates oxidation state; ox refers to cystine (S–S)-containing, "red" to cysteine (SH,SH)-containing; "A" refers to peak A (Met-104 sulfoxide); "B" refers to peak B (Met-104 not oxidized).

** t_G1 and t_G2 are gradient times in min.

*** Very large value of *S* (discarded).

§ Intraday replicates.

weight, amino acid sequence and composition. An obvious explanation is that the conformation of the sorbed protein varies, with a consequent variation in its contact area. We therefore examined the dependence of *Z* values on mutein structure.

Table III shows that *Z* values are unusually large (> 30) for the two cystine

TABLE III

AVERAGE VALUES OF *S*, *Z*, AND log *k'* (55%) FOR PEAK B OF VARIOUS MUTEINS

Values from gradient runs with t_G = 20 and 80 min; values for peak A corrected as described in text (*S*-values divided by 1.06, log *k'* (55%) values add 0.31 units) and averaged with peak B values. *Z* values calculated by eqn. 8.

Mutein	*Cystine (S–S) containing compound*			*Cysteine (SH,SH) containing compound*		
	S	*Z*	*log k' (55%)*	*S*	*Z*	*log k' (55%)*
Ala[1]Cys[125] (parent)	22.1	26.4	0.03	15.7	19.4	0.67
Ala[1]Ser[125]	25.5	29.3	−0.70	19.7	23.2	0.02
desAla[1]Ala[125]	19.3	23.4	0.31	15.6	19.8	0.84
desAla[1]Ala[104]Ser[125]	24.1	27.4	−0.82	21.6	25.5	−0.25
desAla[1]Cys[125]	20.0	23.8	0.05	17.1	21.1	0.61
desAla[1]Ser[125]	22.6	25.8	−0.52	16.2	19.0	−0.01
desAla[1]Ser[58]	36.9*	37.0*	−3.9*	18.7	23.2	0.59
desAla[1]Ser[105]	47.8*	48.7*	−5.0*	17.2	21.0	0.35
desAla[1]Ser[58,105,125]	**	**	**	18.1	21.5	0.04

* Values for compounds with unnatural disulfide-bridge.

** Cystine bond not possible.

derivatives that involve disulfide-bond formation with a cysteine at position 125. Note that removal of a cysteine from either position 58 or 105 destroys the (S^{58}–S^{105}) disulfide bridge in the cystine-containing molecule, and forces an "unnatural" bridge between the remaining two cysteines in the protein (positions 125 and either 58 or 105). It is assumed that the cysteine (SH, SH)-containing molecule will assume a conformation that minimizes exposure of hydrophobic groups to the external water phase, and minimizes its partial-molal free energy. This folding force is strong for the hydrophobic IL-2 molecule, and formation of disulfide bridges will then preferentially occur between cysteine residues that are favorably positioned in this minimum-free-energy conformation. In the case of the IL-2 muteins, removal of one of the two cysteines in the positions favorable to "natural" disulfide formation (58 or 105) then forces disulfide bridging between cysteines in unfavorable positions (125 and either 105 or 58). This would be expected to considerably disrupt the original favored conformation of the cysteine containing molecule, and lower its stability. This in turn favors further unfolding, and should increase the hydrophobic contact surface between protein and the stationary phase. The large increase in Z for the "unnatural" oxidized muteins bears this out.

Previous work characterizing the "unnaturally" oxidized IL-2 protein has shown significant conformational changes which are observable by HPLC. Through RP-HPLC, other researchers have isolated and identified all three cystine disulfide linked isomers of parental sequence IL-2[26,27]. Under the strongly denaturing conditions used to form these isomers, 6 M guanidine hydrochloride at alkaline pH, the cystine disulfides were scrambled and the non-native cystine (S^{105}–S^{125})-containing IL-2 was favored. In contrast, under less denaturing conditions the native cystine (S^{58}–S^{105}) is strongly preferred during oxidation from the cysteine-containing IL-2 molecule[27]. The RP-HPLC elution time of the non-native disulfide isomers, (S^{105}–S^{125}) and (S^{58}–S^{125}) $Ala^{1}Cys^{125}$ (parent) IL-2, were much shorter than for the native cystine (S^{58}–S^{105})-containing IL-2 isomer. These elution shifts were similar to those observed in this study for the "unnaturally" oxidized $desAla^{1}Ser^{58}$ (S^{105}–S^{125}) and $desAla^{1}$(S^{58}–S^{125})Ser^{105} IL-2 muteins. These results suggest that the non-native disulfide isomers of parental sequence IL-2 are correlated with strongly denaturing conditions and that their RP-HPLC retention (and presumably conformation) are similar to the "unnaturally" oxidized Ser^{58} and Ser^{105} IL-2 muteins of the present study.

We have previously shown[22] that methionine sulfoxide at position 104 results in a peak A for the cysteine-containing $desAla^{1}Ser^{58}$(SH^{105},SH^{125}) or des-Ala^{1}(SH^{58},SH^{125})Ser^{105} IL-2 muteins. However, when these compounds are converted to the cystine-containing ("unnaturally" oxidized) forms, a characteristic peak A is absent from the RP-HPLC chromatogram. Thus, the lack of a peak A for the $desAla^{1}Ser^{58}$(S^{105}–S^{125}) and $desAla^{1}$(S^{58}–S^{125})Ser^{105} IL-2 muteins lends additional support to the hypothesis of conformational changes for these "unnaturally" oxidized IL-2 muteins.

Major change in the conformation of the retained protein should affect k' as well as Z, and the data of Table III confirm this: the "unnatural" cystine (S–S)-containing muteins show a decrease in log k' (55%) of 4–5 orders of magnitude compared to their cysteine (SH,SH)-containing counterparts. However, this does not mean that the "unnatural" cystine-containing muteins are always less retained in

TABLE IV

CHANGE IN Z AND log k' (55%) UPON CONVERSION OF CYSTEINE (SH,SH) CONTAINING IL-2 MUTEINS TO THE CORRESPONDING CYSTINE (S–S) CONTAINING COMPOUNDS

Compound	ΔZ	$\Delta \log k'$ *(55%)*
$Ala^{1}Cys^{125}$ (parent)	+ 7.0	−0.64
$Ala^{1}Ser^{125}$	+ 6.1	−0.72
$desAla^{1}Ala^{125}$	+ 3.6	−0.53
$desAla^{1}Ala^{104}Ser^{125}$	+ 1.9	−0.57
$desAla^{1}Cys^{125}$	+ 2.7	−0.56
$desAla^{1}Ser^{125}$	+ 6.8	−0.53
$desAla^{1}Ser^{58}$	+13.8	−4.5
$desAla^{1}Ser^{105}$	+27.7	−5.3
$desAla^{1}Ser^{58,105,125}$	*	*
Averages** ± S.D.	+ 4.7 ± 2.2	−0.59 ± 0.07

* Cystine bond not possible (no cysteines in the molecule).
** Excluding Ser^{58} and Ser^{105} muteins.

RP-HPLC. Examination of their log k_w values (see Table II) shows that these latter compounds are *more* retained (by several orders of magnitude) in the hypothetical case where water is the mobile phase. We will not attempt a further examination of these k' values here. A more detailed analysis of this situation would recognize that the hydrophilic-outside/hydrophobic-inside model of protein structure is somewhat oversimplified.

Table IV summarizes changes in Z and log k' (55%) as a result of cystine-disulfide formation in the various IL-2 muteins of Table III*. Apart from the Ser^{58}, and Ser^{105} muteins, formation of a cystine disulfide results in a fairly constant increase in Z of +4.7 ± 2.2 and decrease in log k' (55%) of −0.59 ± 0.07. Many researchers assume that increased disulfide crosslinking necessarily promotes the stability of the native protein. Whether this is true in a specific instance actually depends on whether denaturation is limited by the kinetics of unfolding, or by the thermodynamic equilibrium between native and denatured states (as well as other factors). For very hydrophobic proteins the formation of a disulfide bridge, even for favorably positioned cysteine groups, may disrupt the thermodynamically-favorable cysteine-containing conformation. This would lead to less stability and greater unfolding upon retention in RP-HPLC. We believe the latter explanation agrees with the constant increase of Z values and decrease of log k' (55%) for cystine (S^{58}–S^{105}) formation in these IL-2 muteins.

Earlier work with IL-2 has shown that the conversion of a single methionine at position 104 to methionine sulfoxide accounts for the transformation of peak B to peak A[22]. This change in the IL-2 molecule results in an increase in methionine polarity, a consistent reduction in reversed-phase retention, and small but consistent increase in Z of + 6% ± 3%. Additional oxidation of the other three methionines in the IL-2 molecule produces only a slight increase in reversed-phase retention[22].

* Other work has shown that the formation of a disulfide bond *per se* has little affect on peptide retention in RP-HPLC[29].

TABLE V

SUMMARY OF EFFECTS OF AMINO ACID SUBSTITUTION IN IL-2 MOLECULE

Amino acid substitution	*Oxidation*		*Δ Z***	*Δ log k' (55%)*
$Cys^{58\ or\ 105} \rightarrow Ser^{58\ or\ 105}$	Cysteine (SH,SH)	(1.5)**	+ 3.1 ± 1.6	−0.05 ± 0.05
$Cys^{58\ or\ 105} \rightarrow Ser^{58\ or\ 105}$	Cystine (S–S)		+17 ± 8	−4.4 ± 0.8
$Cys^{125} \rightarrow Ser^{125}$			+ 2.2 ± 1.5	−0.58 ± 0.15
$Cys^{58,105,125} \rightarrow Ser^{58,105,125}$	Cysteine (SH,SH)	(−4.5)**	+ 0.4	−0.40
$Cys^{125} \rightarrow Ala^{125}$		(−0.4)**	− 0.9 ± 0.6	+0.25 ± 0.01
$Met^{104} \rightarrow Ala^{104}$		(0.5)**	+ 4.1 ± 3.5	+0.41 ± 0.18
$(Met)Ala^{1} \rightarrow desAla^{1}$		(−1.0)**	− 2.2 ± 2.6	+0.09 ± 0.13

* Average values of cysteine (SH,SH) and cystine (S–S) containing muteins reported unless otherwise stated.

** Change in hydrophobicity upon amino acid substitution, Rekker constants[32].

These results suggest that the conformational contribution of the methionine sulfoxide at position 104 is small but constant for all IL-2 muteins which form an A peak.

Consequences of specific substitutions into the IL-2 molecule

Substitution of a different amino acid into the protein backbone can have a significant effect on protein conformation. The magnitude of the effect depends upon both the amino acid substitution and the position of substitution. Table V summarizes these substitutions and lists the resulting changes in Z and log k' (55%). The large standard deviations and lack of correlation with substituent hydrophobicity reflect the unpredictable contribution of a single amino acid residue to the conformational stability of a protein upon reversed-phase binding.

These amino acid substitutions are grouped according to the position of substitution and the cysteine oxidation state of the molecule. Consider the substitution of a cysteine by a serine, a one-atom change of an –SH group to an –OH group. All of these serine substituted muteins show a modest increase in Z which suggests a subtle conformational perturbation. The obvious exceptions are the "unnaturally" disulfide bridged Ser^{58} and Ser^{105} muteins, which exhibit large increases in Z that, we believe, reflect significant change in conformation. The small increase in Z in the $desAla^{1}Ser^{58,105,125}$ mutein (compared to the $desAla^{1}Cys^{58,105,125}$ mutein) is similar to that for a single serine substitution and implies that the conformational effects of cysteine-to-serine substitution are not additive. In contrast, the substitution of alanine at position 125 or removal of (Met)Ala from the hydrophilic N-terminus[33] decreases Z slightly, suggesting that these substitutions may stabilize the protein structure slightly during reversed-phase retention.

Practical applications

One of the goals of the present study was to improve the RP-HPLC separation of protein mixtures, as exemplified by the IL-2 mutein mixtures. It is now known[17,18,29,30] that when S values for two compounds differ, their relative spacing within a gradient chromatogram can be changed by varying flow-rate, F, gradient time, t_G, or column dimensions. Variation of F is simplest and most convenient, and

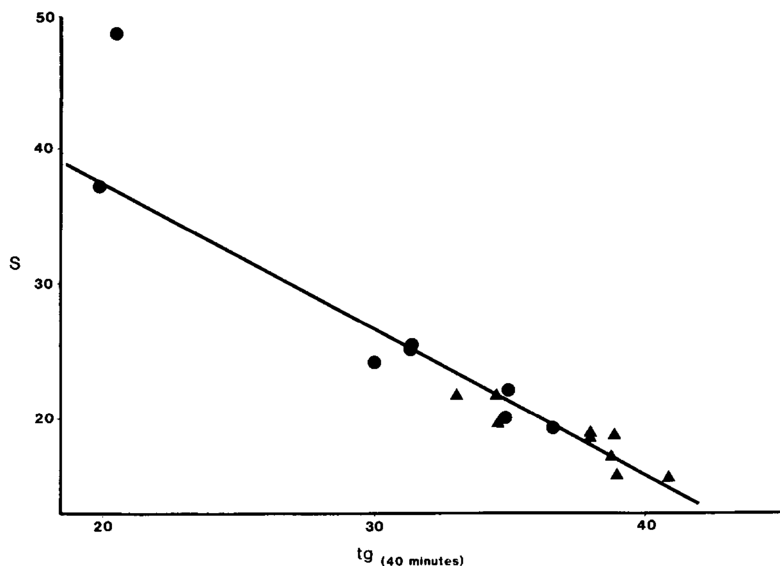


Fig. 5. Correlation of solute S values with retention times in a 40-min gradient. Values from Table III ● = Cystine (S–S)-containing IL-2 muteins; ▲ = cysteine (SH,SH)-containing IL-2 muteins.

this technique has been used to optimize the resolution of various peptide mixtures[31]. The larger the expected variation in S, the greater the likelihood that a change in flow-rate or gradient time will result in resolving previously-overlapped bands. The present example (IL-2), although extreme, shows variation in S by a factor of 3 for compounds of essentially the same molecular weight. This suggests that in some cases

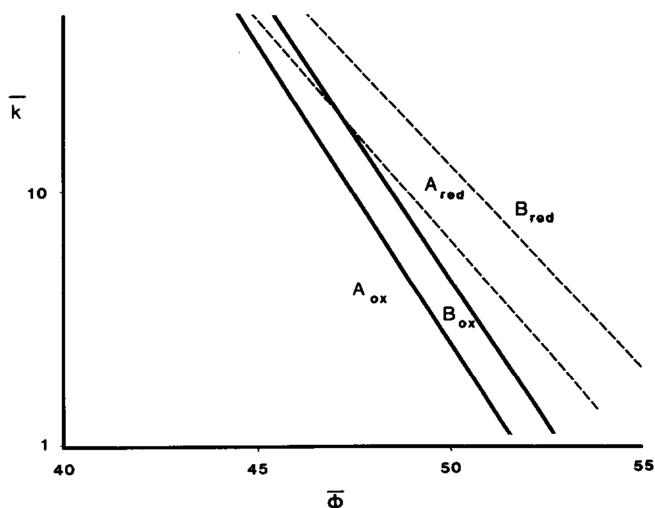
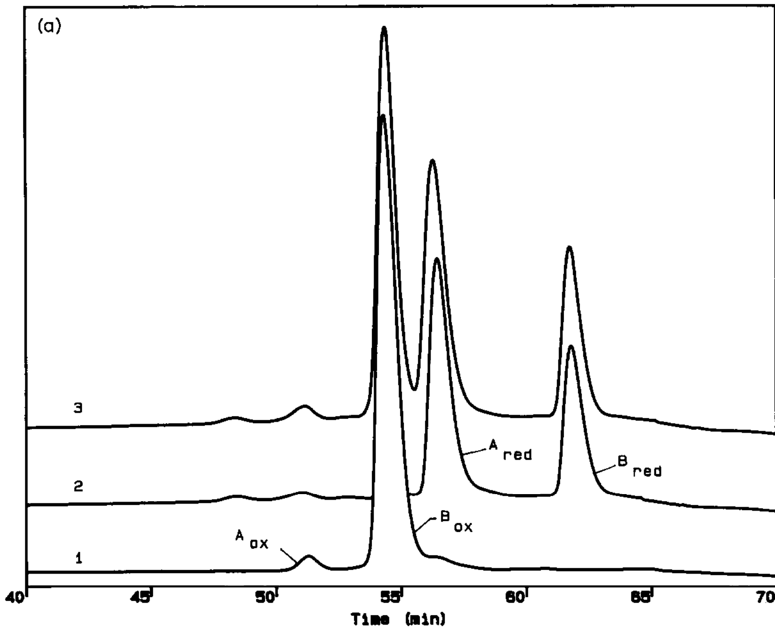


Fig. 6. Predicted retention (log $\bar{k}'$) *versus* percent acetonitrile ($\bar{\varphi}$) in the mobile phase for peaks A and B of cystine-containing (S^{58}-S^{105}; solid lines) and cysteine-containing (SH^{58}, SH^{105}; dashed lines) IL-2. Derived from eqn. 3 and retention data of Table II.



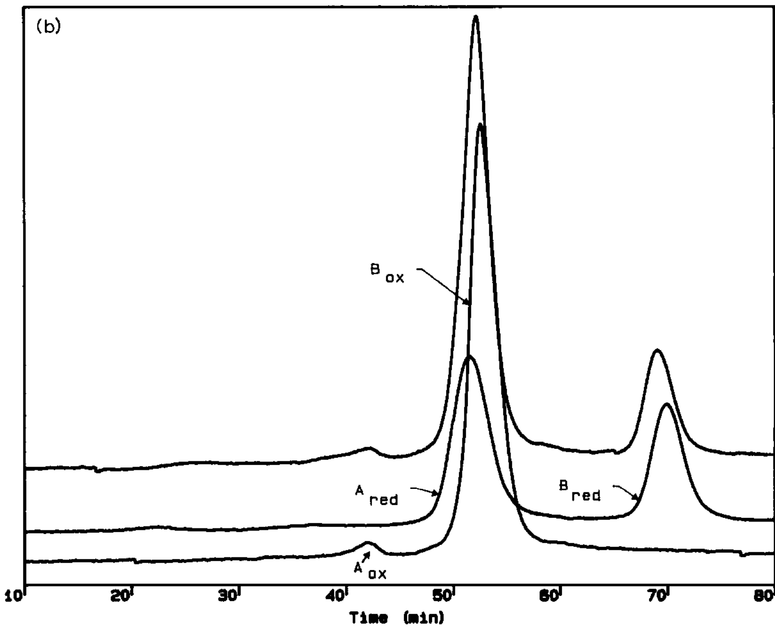


Fig. 7. (a) Chromatograms of; (1) desAla[1](S^{58}–S^{105})Ser[125] IL-2, peak A (MetSO[104]) and peak B (Met[104]); (2) desAla[1] (SH^{58}, SH^{105})Ser[125] IL-2, peak A (MetSO[104]) and peak B (Met[104]); and (3) mixture of all four compounds in Fig. 7a1 and 7a2. Gradient of 41–60% acetonitrile–water (0.1% TFA) in 60 min, at 0.5 ml/min. The IL-2 sample in Fig. 7a1 was oxidized with a 4 *M* equivalent of chloramine T at pH 7.0 at room temperature for 2 min then immediately reduced with 50 m*M* dithiotheitol at pH 8.8 and 60°C for 60 min to generate the IL-2 sample shown in Fig. 7a2. Other conditions described in Experimental. (b) Peak identities are similar to those in (a). Gradient of 44.5–50.8% acetonitrile–water (0.1% TFA) in 80 min, at 2.0 ml/min. Other conditions described in Experimental.

involving mixtures of proteins of similar size, variation of F and/or t_G for gradient runs may result in significant changes in band spacing. Actually, for these IL-2 muteins this is true to only a limited extent. Examination of Fig. 5 shows a strong correlation of S with retention, which indicates that two compounds with similar retention (which we wish to move apart in the chromatogram) will have similar S values. For such compound pairs, a change in flow-rate would have little effect on band-spacing.

Separation of desAla¹Ser¹²⁵ IL-2 mixture

Each IL-2 mutein may be a mixture of four possible compounds: peak A and B of both cysteine and cystine derivatives. The gradient data in Table II can be used to predict the separation of each mutein-mixture as a function of either isocratic (k' *vs.* φ) or gradient ($\bar{k}$ *vs.* $\bar{\varphi}$) conditions. This is illustrated in Fig. 6 for the various derivatives of desAla^{1}Ser^{125} IL-2. It is seen that retention plots for the peaks A and B pair of either the cystine- or cysteine-containing molecule are parallel. This means that a change in conditions (either φ or $\bar{\varphi}$) will have little effect on band-spacing for either cystine- or cysteine-containing pair of compounds. However, the plots for the cystine (S^{58}–S^{105})-containing peak A (MetSO^{104}) and cysteine (SH^{58},SH^{105})-containing peak A (MetSO^{104}) are not parallel, so that the spacing of these bands within the chromatogram will be sensitive to experimental conditions. The same is true for the cystine (S^{58}–S^{105})-containing peak B (Met^{104}) and cysteine (SH^{58},SH^{105})-containing peak B (Met^{104}). The pattern of Fig. 6 for desAla^{1}Ser^{125} IL-2 is similar to that observed for the other muteins in Table II.

Using the plots of Fig. 6, we would predict that at lower values of $\bar{\varphi}$ the cystine-containing peak B will reverse its order of elution with the cysteine-containing peak A. Conversely, at higher values of $\bar{\varphi}$, the separation of cystine-containing peak B and cysteine-containing peak A will be much greater. The chromatograms in Fig.

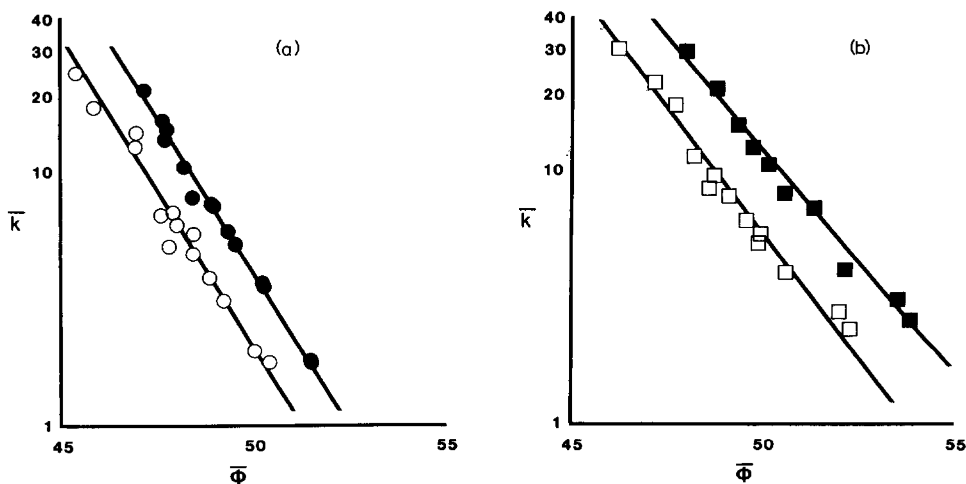


Fig. 8. Relationship of log $\bar{k}$ *vs.* $\bar{\varphi}$ for desAla^{1}Ser^{125} IL-2 over a wide range of $\bar{k}$. Gradient-derived data. (a) ○ = A_{ox} or desAla^{1}(S^{58}–S^{105})MetSO^{104}Ser^{125} IL-2; ● = B_{ox} or desAla^{1}(S^{58}–S^{105})Ser^{125} IL-2. (b) □ = A_{red} or desAla^{1}(SH^{58},SH^{105})MetSO^{104}Ser^{125} IL-2; ■ = B_{red} or desAla^{1}(SH^{58},SH^{105})Ser^{125} IL-2.

7a and b and the retention data of Fig. 8a and b confirm this prediction. At higher values of $\bar{\varphi}$, the cystine-containing peak B (B_{ox}) and cysteine-containing peak A (A_{red}) are clearly separated, while at lower values of $\bar{\varphi}$ they reverse their order of elution. At both of these $\bar{\varphi}$ extremes, the separation of the peak A/B pairs remain similar. Thus, by changing flow-rate and gradient time, we can improve the separation of the cystine-containing pair of peaks A and B from the cysteine-containing pair of peaks A and B. However, we cannot improve the separation of a cystine- or cysteine-containing IL-2 from its corresponding methionine sulfoxide 104 counterpart. This example illustrates both the possibilities and limitations of a change in gradient conditions to affect band-spacing for peptide and protein mixtures, as described more fully in ref. 30.

During the course of optimizing the separation of this IL-2 mutein mixture, additional retention data was obtained covering a broad range of gradient conditions. These data were used to calculate additional values of $\bar{k}$ *vs.* $\bar{\varphi}$ and are plotted in Fig. 8. Within experimental error, the resulting plots are linear, which confirms the utility of this technique over a wide range of gradient conditions.

CONCLUSIONS

The retention of proteins and other molecules in RP-HPLC is believed to occur by a displacement process. Stoichiometry factors Z or S can be determined from chromatographic retention data, and these are believed to measure the relative contact area of solute and bonded-phase surface. Thus, as the protein molecule unfolds from its original (native) state, its contact area increases and values of S or Z increase. We believe that the measurement of protein Z values can be useful in defining protein conformation during RP-HPLC.

In the present study, 30 IL-2 muteins were chromatographed using standard RP-HPLC gradients in acetonitrile–water with 0.1% TFA. It was found that values of Z varied by 2.5-fold, suggesting that the conformation of the retained molecule varies considerably among this series of compounds (molecular weight and amino acid composition are also believed to affect values of Z, but these parameters are virtually identical for these IL-2 muteins). It was possible to rationalize these variations in Z in terms of protein structure.

The IL-2 molecule is quite hydrophobic, and it is believed that this stabilizes the structure of the folded molecule, both in solution and on the stationary phase. Formation of a disulfide bridge should disrupt the most stable native structure, and formation of an "unnatural" bridge (in muteins where a cysteine at position 58 or 105 is missing) should be particularly destabilizing. In agreement with these expectations, it is found that average values of Z increase by about 20% upon formation of the "natural" (S^{58}–S^{105}) disulfide. Oxidation to form the "unnatural" cystine results in an almost 2-fold increase in Z. That is, destabilization of the mutein molecule through non-native disulfide formation results in greater denaturation upon reversed-phase retention, and a corresponding increase in the value of Z. Less hydrophobic peptides should show such changes to a lesser degree, due to their more complete denaturation during RP-HPLC separation. Oxidation of the Met (104) amino acid to its sulfoxide gave a small increase in Z plus a decrease in retention. These changes were relatively constant from one mutein to another (and for either

cystine or cysteine containing muteins), suggesting a small but consistent conformational destabilization as a result of this substitution. Other changes in Z were observed upon various substitutions of amino acids into the parent IL-2 molecule. These changes were generally small, sensitive to the position of substitution, and not easily correlated with the hydrophobicity of the amino acids involved. However, the present study shows that minor changes in protein composition at some positions can lead to significant changes in values of Z (and S). This suggests that the technique of varying flow-rate for gradient separation will often lead to changes in band spacing and separation of mixtures of peptides or proteins.

As a practical application of the present study, the separation of the cysteine and cystine forms of peaks A and B of desAla^{1}Ser^{125} IL-2 could be predicted as a function of experimental conditions, based on measurements of values of S. It was possible to obtain an even spacing of the four species with one set of gradient conditions, and to obtain reversal of elution order for two of the species with another set of conditions. This example demonstrates that optimization of separations such as this can be achieved by calculation, rather than trial-and-error methods.

Previous work[21] has shown that isocratic retention parameters, such as Z and S, can be determined reliably from two or more gradient runs. The present study further confirms the potential accuracy of this procedure for both small and large molecules. Specifically, it was shown here that values of S or Z can be measured with a precision of 6% R.S.D. This appears adequate for the assessment of protein conformation during reversed-phase separation.

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