Anal. Chem. **2002,** *74,* 3662−3669

Controlling Deuterium Isotope Effects in Comparative Proteomics

Roujian Zhang, Cathy S. Sioma, Robert A. Thompson, Li Xiong, and Fred E. Regnier*

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

This paper focuses on identifying structural features responsible for resolution of heavy isotope coded peptides during reversed-phase chromatography. This was achieved by using labeled coding agents that varied in structure, number of deuterium atoms, placement of deuterium in the coding agent, and the functional group targeted by the reagent. Six coding agents were examined. Deuterated versions of the coding agents studied included succinic anhydride-$^2H_4$, acetic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_3$, propionic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_5$, pentanoic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_9$, [3-(2,5-dioxopyrrolidin-1-yloxycarbonyl)-propyl]-trimethylammonium chloride-$^2H_9$, and the commercial ICAT-$^2H_8$ reagent. It was found that these labeling agents vary widely in both their absolute and relative contribution to the chromatographic isotope effect. Relative effects were evaluated by normalizing resolution for the number of deuterium atoms in the derivatized peptide. The single, most dominant effect was the placement of deuterium atoms relative to hydrophilic functional groups in the coding agent. It was concluded that the probability of a deuterium atom interacting with the stationary phase of a reversed-phase chromatography (RPC) column and impacting resolution is greatly diminished by placing it adjacent to a hydrophilic group, as explained by solvophobic theory. But peptide size and coding agent size were also seen to correlate inversely with the magnitude of the isotope effect. This effect was explained as being due to the relative size of the coding agent versus that of the coding agent−peptide conjugate.

Although "proteomics" is a recently coined term,[1] comparative proteomics has been practiced for at least three decades. Among the earliest studies with 2-D gel electrophoresis was one involving the examination of thousands of proteins in blood.[2−4] These reports and many since depended on resolution of complex protein mixtures with 2-D gels and differential staining to compare samples and recognize differences. The problem with these classical methods is that they were labor-intensive, quantification was poor, and it was difficult to identify spots thought to be important.

With the advent of huge DNA and protein sequence databases, there is an increasing dependence on mass spectrometry to identify proteins and peptides obtained from separation systems. The speed and resolution of mass spectrometers is shifting the focus in proteomics toward more rapid delivery of peptide mixtures to mass spectrometers and obtaining quantitative, as well as qualitative data during mass analysis.

Quantification of peptides has been greatly aided by the development of in-vitro and in-vivo heavy-isotope coding methods for peptides and proteins in complex mixtures.[5−19] The method of choice is to differentially tag tryptic peptides from two different samples with isotopically distinct forms of a coding agent, and after mixing, components of the mixture are chromatographically or electrophoretically fractionated, and the abundance ratio of the coded peptide isoforms is determined by mass spectrometry. The relative concentration of peptides thus obtained is used to evaluate protein expression and regulatory changes as a function of cellular stimuli.[7]

A problem with this strategy is that coded peptides varying in numbers of heavy atoms can be partially or even completely resolved during reversed-phase chromatography (RPC), particularly in the case of deuterium labeling. This presents several

* Corresponding author. E-mail: fregnier@purdue.edu.

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10.1021/ac025614w CCC: $22.00
Published on Web 07/04/2002

troublesome issues. The most serious is that the observed abundance ratio varies continuously across a chromatographic peak, and it is difficult to determine the true abundance ratio of an analyte quickly. Abundance ratios in this case must be computed from comparative area measurements using extracted ion chromatograms. This means that the analyte peak will have completely eluted from the LC−MS system before it is known whether the peptide has changed in concentration and is of interest.[15] Moreover, ionization efficiency can vary across a peak as a result of sample matrix contributions and impact accuracy.[20]

This separation problem is equally troubling in MALDI-MS in which it is necessary to use collected fractions for spectral analyses. Chromatographic fractions may be enriched in one isotopic isoform over the other and differ widely in matrix components, both of which compromise abundance ratio measurements. Clearly, minimizing chromatographic resolution of peptide isoforms would improve measurement accuracy and enable real-time measurements.[11,15,16,20,21,24] Rapid determination of abundance ratios is of great interest, because it is a critical element in on-line, intelligent data acquisition and analysis (IDA),[21] which may also be referred to as real-time data-dependent analysis (DDA).

It has recently been shown that there is little or no resolution of peptide isoforms during reversed-phase chromatography when carbon-13-labeled succinic anhydride is used as the coding agent.[21] But carbon-13-labeled coding agents are more expensive and are generally larger. This is particularly true when the difference between isotopically coded isoforms is 8 or more atomic mass units, as needed with lower resolution instruments or large peptides. Larger tagging agents can generate fragmented ions that may interfere with MS/MS sequencing.[16] In contrast, deuterium labeling has the attractive features of being less expensive and simpler to use in the preparation of coding agents in addition to allowing smaller coding agents.

The objective of the work described here was directed at identifying structural features responsible for deuterium isotope effects in reversed-phase separations and ways to minimize the fractionation of deuterated isoforms. This was achieved by using labeling agents that varied in structure, number of deuterium atoms, placement of deuterium in the coding agent, and the functional group targeted by the reagent.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), cytochrome *c*, HPLC grade acetonitrile (ACN), *N*-hydroxysuccinimide (NHS), succinic anhydride, dithiothreitol (DTT), iodoacetic acid (IAA), urea, tris(hydroxymethy)aminomethane (Tris base), tris(hydroxymethyl)-aminomethane hydrochloride (Tris acid), and calcium chloride were purchased from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid (sequanal grade) was acquired from Pierce (Rockford, IL). Sequencing grade modified trypsin was supplied from Promega (Madison, WI). Succinic anhydride-$^2H_4$ was obtained from Isotec (Miamisburg, OH). The ICAT kit was purchased from Applied Biosystems (Framingham, MA). The C18 column (2.1 × 250 mm) was purchased from Vydac (Hesperia, CA). Double-diionized water (ddI H_2O) was produced by a Milli-Q Gradient A10 System from Millipore (Bedford, MA).

2,5-Dioxopyrrolidin-1-yl Esters. The 2,5-dioxopyrrolidin-1-yl esters of acetic acid, propionic acid, pentanoic acid, and 4-trimethylammonium butyric acid were prepared according to a procedure described by Staros.[22]

Proteolysis of BSA and Cytochrome *c*. BSA and cytochrome *c* (5 mg) samples were digested by first reducing in 1 mL of 0.2 M Tris buffer (pH 8.5) containing 8 M urea and 10 mM DTT. After 2 h incubation at 37 °C, iodoacetic acid was added to a final concentration of 20 mM and the mixture was incubated in darkness on ice for an additional 2 h. The solution was then diluted with 0.2 M Tris buffer to a final urea concentration of 2 M. Sequencing grade trypsin was added (2% of enzyme to total protein; w/w) and the mixture was incubated for 8 h at 37 °C. Digestion was stopped by freezing the mixture in liquid nitrogen for 10 min.

Derivatization of Peptides. A 50-fold molar excess of derivatization reagent (i.e., succinic anhydride-$^2H_0$ and -$^2H_4$, acetic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_0$ and -$^2H_3$, propionic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_0$ and -$^2H_5$, pentanoic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_0$ and -$^2H_9$, or [3-(2,5-dioxopyrrolidin-1-yloxycarbonyl)-propyl]-trimethylammonium chloride-$^2H_0$ and -$^2H_9$) was added individually to peptide mixtures of experimental and control samples, and the reaction was allowed to proceed for 2 h at room temperature. Peptides were also derivatized with ICAT-$^2H_0$ and $^2H_8$ as described by the supplier.

Reversed-Phase Elution of Isotopically Labeled Peptides. Isotopically labeled peptide mixtures were separated by gradient elution from a Vydac C18 column (2.1 mm × 250 mm) on an Integral Micro-Analytical Workstation (Applied Biosystems, Framingham, MA). The C18 column was equilibrated using 100% mobile phase A (0.01% TFA in ddI H_2O) at a flow rate of 250 μL/min for 2 column volumes (CV). Isotopically labeled peptide mixtures (2 nmol) were injected and eluted at a flow rate of 250 μL/min in a linear gradient ranging over 60 min from 100% mobile phase A to 60% mobile phase B (95% ACN/0.01% TFA in ddI H_2O). At the end of this period, a new linear gradient was applied in 10 min from 60% B to 100% B at the same flow rate. The gradient was then held at 100% mobile phase B for an additional 10 min. Throughout the analysis, an on-line UV detector set at 214 nm was used to monitor separation of the peptide mixtures. The peptides were simultaneously monitored with an ESI-MS by directing 10% of the flow to the ion source.

ESI-MS Analysis. Mass spectral analyses were performed using a QSTAR workstation (Applied Biosystems, Framingham, MA) equipped with an ion-spray source. All spectra were obtained in the positive ion mode of TOF at a sampling rate of one spectrum every 2 s. During the LC−MS acquisition, masses were scanned from m/z 300 to 1800.

RESULTS AND DISCUSSION

Resolution of Isotopically Coded Peptides. Six deuterium-labeled coding agents were examined in this work, including succinic anhydride-$^2H_4$ (a), ICAT-$^2H_8$ (b), acetic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_3$ (c), propionic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_5$ (d), pentanoic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_9$ (e),

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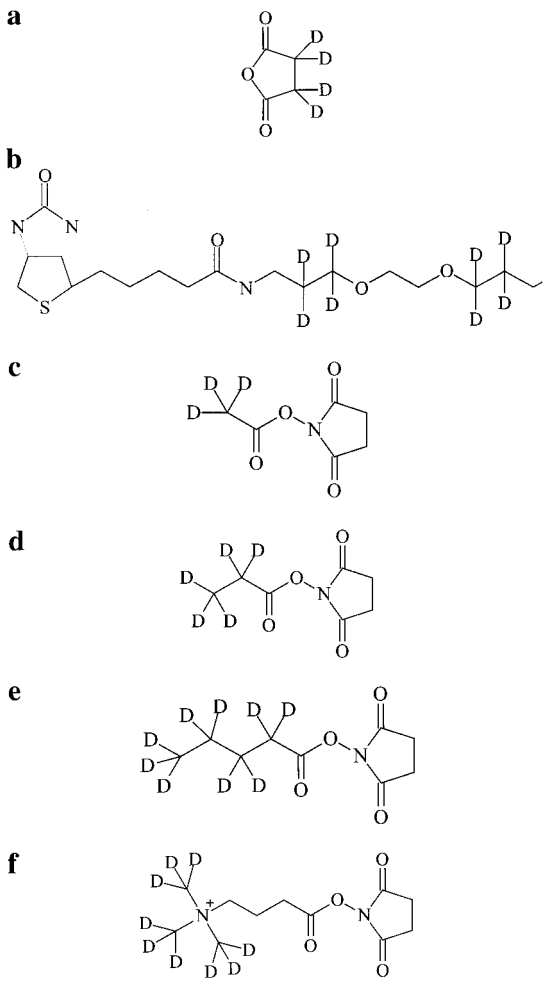

Figure 1. Structures of derivatizing agents (deuterated version): (a) succinic anhydride-$^2H_4$, (b) ICAT-$^2H_8$, (c) acetic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_3$, (d) propionic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_5$, (e) pentanoic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_9$, and (f) [3-(2,5-dioxopyrrolidin-1-yloxycarbonyl)-propyl]-trimethylammonium chloride-$^2H_9$.

and [3-(2,5-dioxopyrrolidin-1-yloxycarbonyl)-propyl]-trimethylammonium chloride-$^2H_9$ (f) as seen in Figure 1. The four *N*-hydroxysuccinimide (NHS)-activated carboxylic acids (Figure 1c−f) and succinic anhydride (Figure 1a)-derivatized primary amine groups on the ϵ-NH_2 group of lysine residues and α-NH_2 groups formed during proteolysis. Amino terminally blocked peptides that contain no lysine residues were not derivatized by these reagents.

The technique of coding functional groups formed during the course of proteolysis for the purpose of quantification has come to be known as global internal standard technology (GIST).[7] Differential labeling of tryptic digests from control and experimental samples with isotopically different forms of GIST reagents produced peptide isoforms that vary in molecular weight by some multiple of the number of deuterium atoms in the GIST reagent. The multiple in the case of C-terminal lysine-containing peptides was generally two, because both the amino terminus of the peptide and the free ϵ-amine group of lysine were derivatized with the coding agent. In this case, mass spectra of the coded peptides appear as a double cluster of ions separated by twice the number of atomic mass units (amu) seen with C-terminal arginine-containing peptides. The mass differences (M) between the isotopically coded peptides are determined by following equation,

$$M = \frac{mnd}{z} \tag{1}$$

where m is the mass increment caused by one isotope atom (for example, $m(^2\mathrm{H}) = 1$, $m(^{18}\mathrm{O}) = 2$, etc.), n is the number of isotopes in the coding agents, d is the number of derivatizable groups in the peptide, and z is the charge of the peptides in mass spectra.

The sixth derivatizing agent examined was the commercial ICAT reagent (Figure 1b).[5] This reagent is a sulfhydryl-directed alkylating agent composed of iodoacetate attached to biotin through a coupling arm. Isotopic isoforms of this reagent are differentially labeled in the coupling arm (Figure 1b). During the course of reducing and alkylating proteins in preparation for proteolysis, the ICAT reagent was used to alkylate cysteine residues in proteins. After differential labeling of control and experimental samples with the $^2H_0$ and $^2H_8$ versions of the ICAT reagent respectively, the samples were mixed and digested with a proteolytic enzyme. Biotinylated, cysteine-containing peptides were selected from digests with avidin, and after release from the affinity column, the selected peptides were further resolved by reversed-phase chromatography and the relative concentration of the isotopic isoforms quantified by mass spectrometry. ICAT reagent can be used to quantify changes only in a proteome that involves cysteine-containing peptides.

Identifying structural features of coding agents that impacted the separation of isoforms required the determination of peptide resolution. Resolution (R) is generally defined as

$$R = \frac{\Delta}{W_{1/2}} \tag{2}$$

where Δ is the chromatographic separation between deuterated and nondeuterated isoforms of a peptide, and $W_{1/2}$ is the average full peak width at half-maximum (fwhm). Another form of this equation expresses peaks in terms of the intergral of eluted analyte versus time and resolution as the shift in time necessary to cause the two curves to overlap.[20]

As the number of deuterium atoms in labeling agents increased from three in $^2H_3$-acetate to five in $^2H_5$-propionate through nine in $^2H_9$-pentanoate, resolution of the deuterated and nondeuterated isoforms of peptides increased as well (Figure 3c). This meant that analyses of structural contributions to chromatographic behavior would be most easily achieved by normalizing resolution for deuterium content, particularly in the case of peptides derivatized at multiple sites. Resolution was normalized using the equation

$$R_s = \frac{R}{D_a} \tag{3}$$

where R is the resolution between deuterium- and nondeuterium-enriched isoforms of a coded peptide, D_a is the total number of deuterium atoms incorporated into the deuterated peptide during derivatization, and R_s is referred to as specific resolution.

Cytochrome *c* was chosen as a model protein for analysis of isotope effects, because it produced many peptides that would be

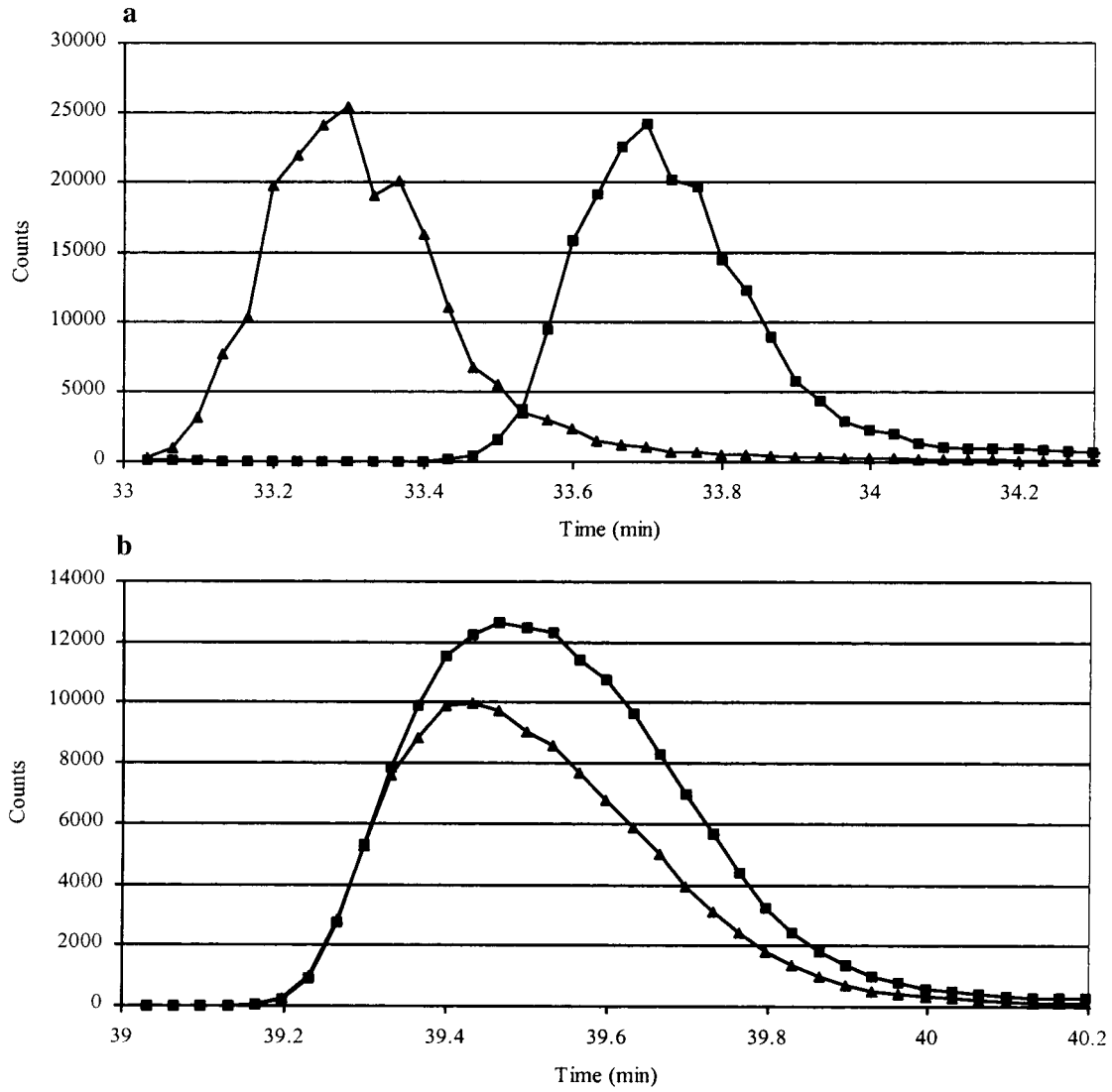

Figure 2. Fractionation of isotopically labeled peptides during reversed-phase chromatography. (a) The peptide (KGER) was derivatized with both pentanoic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_0$ (■) and $^2H_9$ (▲). Eighteen deuterium atoms were incorporated into the peptide. The deuterated (▲) and nondeuterated peptides (■) were completely separated in reversed-phase chromatography ($R = 1.7$). (b) The peptide (EDLIAYLK) was derivatized by [3-(2,5-dioxopyrrolidin-1-yloxycarbonyl)-propyl]-trimethylammonium chloride-$^2H_0$ (■) and $^2H_9$ (▲). Eighteen deuterium atoms were incorporated into the peptide. The deuterated (▲) and nondeuterated peptides (■) were only slightly separated in reversed-phase chromatography ($R = 0.077$).

derivatized by all of the reagents being studied except ICAT. Bovine serum albumin (BSA) was used to study resolution and specific resolution with the ICAT reagent. These two proteins provide a set of peptides that vary widely in hydrophobicity and mass range. Many proteins produce tryptic fragments larger than those obtained from cytochrome *c*, but it will be seen below that chromatographic resolution of isotopic isoforms is usually not an important issue in large peptides.

The Impact of Coding Agent Structure on Isotope Effects. The extremes in resolution of coded peptides were seen in the comparison of pentanoate (Figure 1e) with trimethyammonium butyrate (Figure 1f) derivatization. Examples of the resolution of deuterated and nondeuterated peptides derivatized with pentanoate and trimethyammonium butyrate are seen in Figure 2a and b, respectively. Pentanoate-derivatized peptides showed roughly 10 times greater resolution in most cases (Figure 3a−b). This is surprising in view of the fact that both reagents contain 9 deuterium atoms and trimethylammonium butyrate is slightly larger. Clearly, the dramatic difference in isotope effect seen between these two reagents is due to more than the number of deuterium atoms in the coding agents. Structure must play an important role. The major difference in the structure of these two reagents is in the presence of a quaternary amine group in trimethylammonium butyrate and the clustering of deuterium atoms around the charged nitrogen.

Separations in reversed-phase chromatography are generally explained by solvophobic theory.[23] According to this theory, a quaternary ammonium group would be expected to interact much more weakly with the hydrophobic stationary phase of a reversed-phase chromatography (RPC) column than the aliphatic chain of pentanoic acid. The essential elements of solvophobic theory are that (1) there is a strong driving force in solutions of high surface

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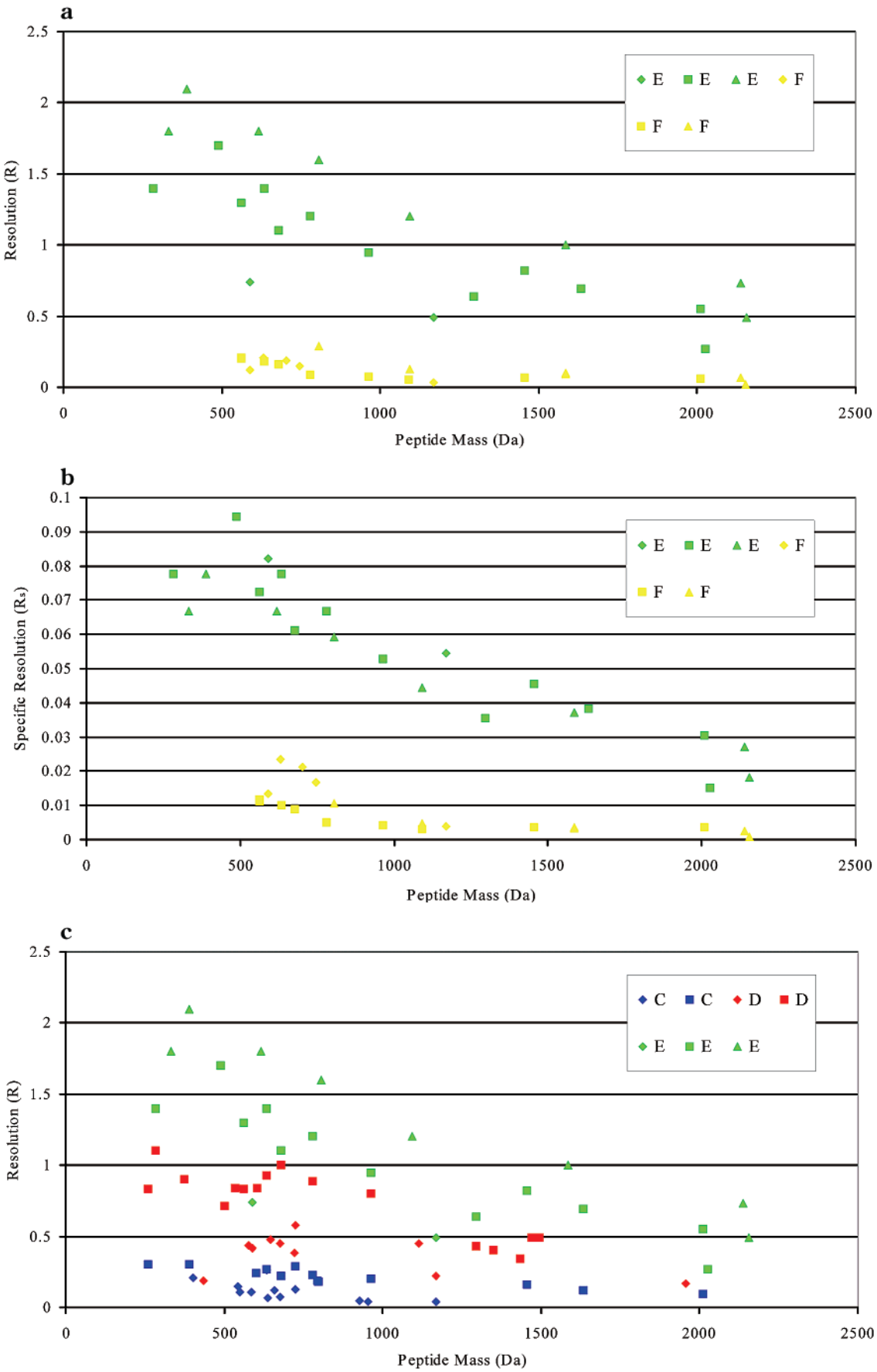
a
Resolution (R)
Peptide Mass (Da)
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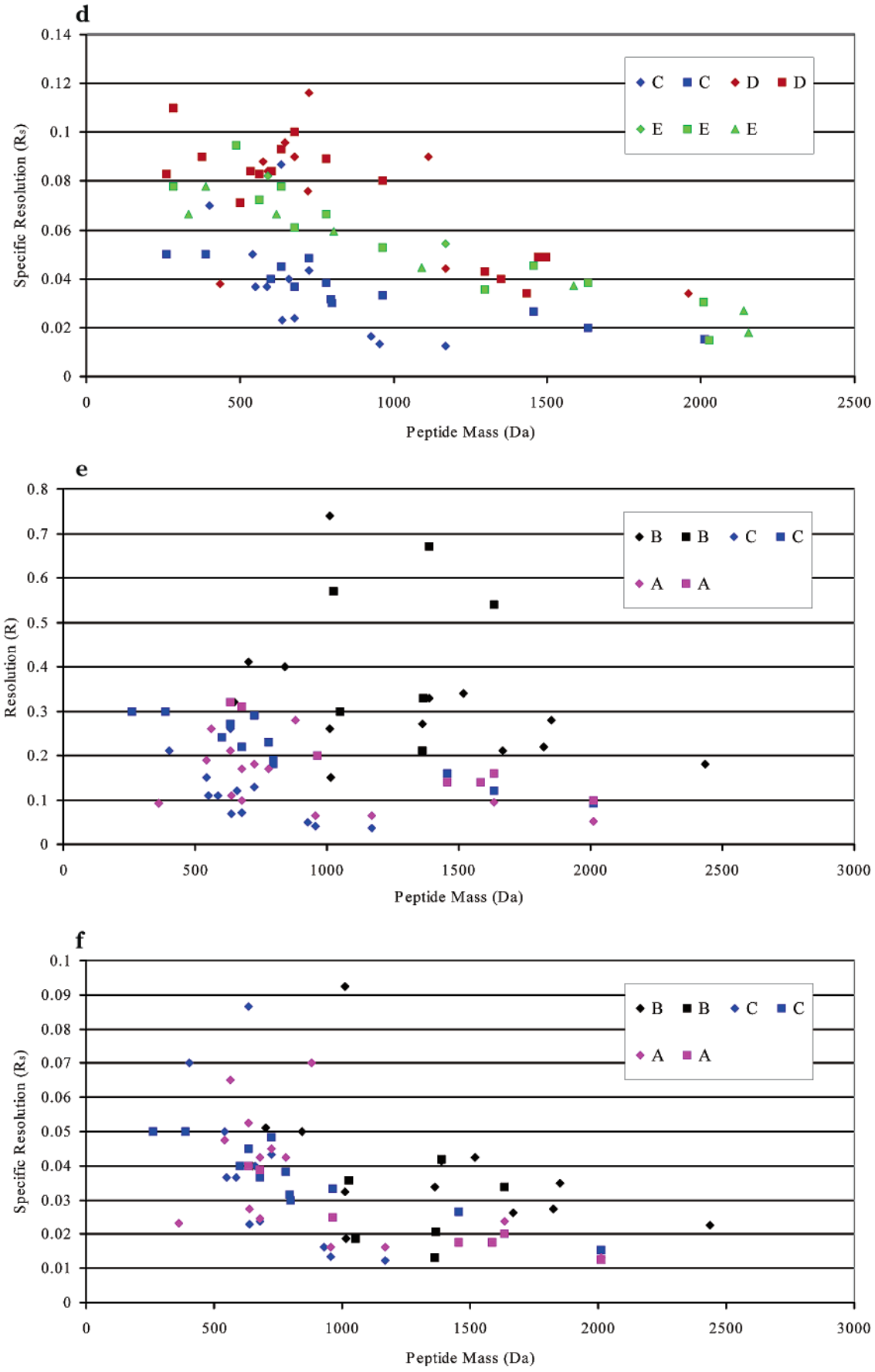

Figure 3. Comparison of resolution (R) and specific resolution (R_s) of peptides having different numbers of derivatizable groups. Reagents A−E used in these experiments are as indicated in Figure 1. The number of sites derivatized is indicated by the symbols ♦ = 1, ■ = 2, ▲ = 3 (those peptides have two lysine residues resulting from trypsin miscleavages).

tension, such as the polar mobile phases used in RPC, to exclude molecules that do not solvate well and (2) nonpolar groups of analytes are driven by polar solutions to interact with the hydrophobic octadecyl stationary phase of an RPC column to minimize the hydrophobic contact area of both with RPC mobile phases. This means that deuterium atoms in a hydrophobic domain of a coding agent would be forced to interact with the stationary phase of an RPC column and because deuterium atoms are less hydrophobic that hydrogen atoms, deuterated species would be less strongly retained. Coding agents that are less likely to interact with the stationary phase would thus be less likely to cause peptide resolution on the basis of their deuterium content than those with a high probability of contact. Moreover, grouping deuterium atoms around hydrophilic moieties should minimize both their interaction with the stationary phase and minimize the isotope effect.

Data from pentanoate and trimethyl butyrate fit this hypothesis (Figure 3a−b). So does the behavior of the ICAT and succinate reagents (Figure 3e−f). Specific resolution (R_s) with the ICAT and succinate reagents was higher than those with trimethylammonium butryrate, but much lower than with pentanoate. This would be predicted by the hypothesis. Deuterium atoms in the ICAT reagent are near ether and amide linkages. These groups are much more polar than the alkyl chain in pentanoate but are less polar than the quaternary amine in trimethylammonium butyrate. For this reason, deuterium atoms in the ICAT and succinate reagents will have a lower probability of interacting with the hydrophobic stationary phase than those in the pentanoate chain, but a higher probability than deuterium atoms in trimethylammonium butyrate. The position of deuterium atoms relative to hydrophilic groups played a role in the deuterium isotope effect with all the species examined.

The behavior of aliphatic acid coding agents was also used to test the hypothesis. As expected, resolution (R) was directly proportional to the number of deuterium atoms in the coding agent (Figure 3c) and deuterated species eluted from columns first. But more important was the fact that there was little difference in the specific resolution (R_s) of pentanoate and propionate, whereas values for acetate were only slightly lower (Figure 3d). These data show that deuterium atoms in alkyl chains of coding agents interact strongly with stationary phases and make a large contributions to resolution. The fact that the R_s is very similar for all of the aliphatic acids is a further validation of the solvophobic hypothesis.

The Impact of Peptide and Coding Agent Size on Isotope Effects. Peptide molecular weight in Figure 3 is of peptides before derivatization. It is seen in Figure 3c−d that resolution decreased as peptide mass increased in the cases of pentanoate, propionate, and acetate. The isotope effect was most obvious in the larger pentanoate reagent with the greater number of deuterium atoms and decreased with the size of the coding agent and number of deuterium atoms. This phenomenon is thought to be due to the size of the peptide-coding agent conjugate relative to the size of the coding agent alone. As peptide size increases, the relative contribution of the coding agent to retention and resolution would be expected to decrease. The same peptide size contribution was seen in all cases, but the magnitude of the effect decreased and became less pronounced as specific resolution grew smaller (Figure 3a−f).

The impact of peptide size among reagents that showed a substantial isotope effect was least distinct with the ICAT reagent (Figure 3e−f). ICAT has a molecular weight of 442. In the case of peptides derivatized at two sites, their molecular weight would increase by 884 amu. Peptides in this study ranged from 500 to 2500 in molecular weight before derivatization. This means that ICAT derivatization could contribute 15−64% of the mass of derivatized peptides. It is to be expected that the contribution of the ICAT reagent with increasing peptide size would be smaller. Although increasing the size of a coding agent minimizes the isotope effect, particularly with low-molecular-weight peptides, this probably is not a good strategy. Large coding agents also generate more fragmented ions and can cause interpretation problems during MS/MS sequencing.[16]

General Trends. The hydrophobicity of the domain into which heavy isotopes are incorporated is by far the most important indicator of whether a coding agent will exhibit a deuterium isotope effect during reversed-phase chromatography. When a deuterium atom is near a polar functional group, it will have a smaller R_s value and make a smaller contribution to resolution. Minimizing R_s is even more important than the total number of deuterium atoms in the derivatizing agents. For example, acetate with three deuterium atoms (Figure 3c) showed a larger isotope effect than trimethylammonium butyrate with nine deuterium atoms (Figure 3a). Grouping deuterium atoms around polar functional groups that show little affinity for the stationary phase dramatically reduces the isotope effect during reversed-phase chromatography.

A less effective way to control the isotope effect is to minimize the number of deuterium atoms in the coding agent, but it works. Although the absolute resolution of $^2H_3$- and $^1H_3$-acetate is much smaller than that with the $^2H_8$- and $^1H_8$-ICAT reagent (Figure 3e), the impact of a single deuterium atom on resolution, as judged by R_s, is actually only slightly higher with ICAT (Figure 3f). The issue becomes how many deuterium atoms are needed for coding. With peptides ranging from 500 to 3000 in molecular weight and high-resolution mass spectrometers, three deuterium atoms is adequate. A modified ICAT reagent containing three deuterium instead of eight has been reported to reduce the deuterium isotope effect.[24]

Yet another way to minimize isotope effects would be to minimize the number of derivatization sites. Derivatization at multiple sites amplified the isotope effect in all cases (Figure 3a,c,e). This suggests that differential derivatization of the relatively abundant carboxyl groups in peptides with $^2H_3$- and $^1H_3$-methanol[19] will also be likely to show isotope effects.

Finally it was seen that with peptides of 2500 Da or higher molecular weight, the isotope effect was minimal with all reagents examined except pentanoate. The deuterium isotope effect is really only an issue with small- to intermediate-sized peptides.

CONCLUSIONS

It can be concluded from these studies that the reversed-phase chromatographic fractionation of peptides derivatized with coding agents differentially labeled with deuterium can be minimized to acceptable values by grouping deuterium around polar func-

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tional groups in the coding agent and using smaller numbers of deuterium atoms in coding agents.

ACKNOWLEDGMENT

The authors gratefully acknowledge support from the National Institute of Health (GM 59996). The authors also thank Peiran Liu for providing acetic acid 2,5-dioxopyrrolidin-1-yl ester-$^2H_0$ and -$^2H_3$.

Received for review March 5, 2002. Accepted May 10, 2002.

AC025614W