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Removal of the fluorescent 4-(aminosulfonyl)-2,1,3-benzoxadiazole label from cysteine-containing peptides

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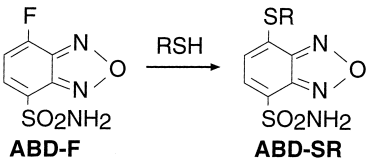
Abstract

The complete removal of the fluorescent cysteine derivative 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) from an intact protein has not been demonstrated even after extended treatment with a reducing agent. It has been suggested that this may be due to incomplete denaturation under the conditions employed. We were interested in investigating this phenomenon utilizing small peptides containing individual ABD-labeled cysteine residues. After incubating the fluorescent peptides in the presence of a reductant, it was shown that the ABD label could be completely removed from all of the cysteine-containing peptides investigated. Therefore, delabeling irreversibility is due to residual structure in proteins. Electrospray ionization mass spectrometry (ESI-MS) was used to determine the molecular mass of each peptide after removal of the ABD label. The ESI-MS data were consistent with the generation of a free sulfhydryl. The generation of the free sulfhydryl was further substantiated when a delabeled peptide was completely relabeled with ABD-F in the absence of reductant.

Keywords: Derivatization, LC; 4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; Cysteine; Peptides; Sulfhydryl groups

1. Introduction

Incorporation of fluorescent labels on specific residues of proteins or peptides offers a convenient mode of detection in a variety of analytical techniques. Several reagents are available for fluorescently labeling cysteine residues and include monobromobimane [1], fluorescein maleimide [2], 5-N-[(iodoacetamidoethyl)amino]naphthalene-1-sulfonic acid (5-I-AEDANS) [3], 5-iodoacetamido-fluorescein [3] and 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [4]. All of these reagents appear to react similarly in their ability to label cysteine residues, however, a possible advantage in the use of ABD-F is that it has been shown [5,6] to be a quantitative method for fluorescently alkylating cysteine according to [4]:



ABD-F **ABD-SR**

Recent work [7] has indicated that the fluorescent ABD–cysteine labeling reaction is reversible which provides the potential for the regeneration of a free sulfhydryl for further modification or reformation of a disulfide. Although the stability of the ABD–cysteine label under several conditions has been shown previously [5,7], detailed studies using bovine serum albumin (BSA) showed the formation of the

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ABD–cysteine adduct to be reversible in the presence of a reducing agent [7]. However, approximately 15–20% of the ABD label was not displaced. In addition, individual cysteines were not analyzed after removal of the ABD label to support the reformation of the free sulfhydryl.

In the present study, we have investigated the reversibility of the ABD–cysteine fluorescent label from small peptides containing individual cysteine residues. The data indicated that, in contrast to BSA, the ABD label could be completely removed from all of the peptides investigated. Further, electrospray ionization mass spectrometry (ESI-MS) indicated that the molecular mass of the cysteine derivative generated after removal of the ABD label was consistent with a free sulfhydryl. The generation of the free sulfhydryl was further substantiated when a delabeled peptide was relabeled with ABD-F in the absence of reductant.

2. Experimental

2.1. Materials

All reagent grade chemicals and buffer components including N-tris[hydroxymethyl]-methylglycine (tricine) were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma (St. Louis, MO, USA). Lysyl Endopeptidase (LysC) and ABD-F were obtained from Dojindo (Japan) through Wako Chemicals (Dallas, TX, USA). Tributyl phosphine (TBP) was purchased from Aldrich (Milwaukee, WI, USA). Dithiothreitol (DTT) was obtained from Bio-Rad Labs. (Richmond, CA, USA). The Endoproteinase AspN (AspN) was purchased from Boehringer Mannheim (Indianapolis, IN, USA). The ABD-labeled cysteine-containing peptides were generated from the following Amgen purified proteins: stem cell factor [8] (peptides 1–8 listed in Table 1), granulocyte colony stimulating factor [9] (peptides 9 and 10 listed in Table 1) and megakaryocyte growth and development factor [10,11] (peptides 11–14 listed in Table 1). All sequence analyses were performed on a Hewlett-Packard G1005A protein sequencer and all amino acid analyses were performed on a Beckman 640 amino acid analyzer.

2.2. Peptide mapping and labeling of cysteine residues with ABD-F

Either LysC and/or AspN were used to treat approximately 200–250 μg of each protein utilized for generating cysteine containing peptides. For the EndoLysC treatments, the protein samples were diluted into 100 μl of 8 *M* urea, 10 μl of 1 *M* Tris–HCl, pH 8.5 and 4 μg of LysC (from a 1 mg/ml stock solution in 10 m*M* Tris–HCl, pH 8.5). The total volume was adjusted to 200 μl with distilled water, and the proteolytic digestion was

Table 1
The amino acid sequences for the purified ABD-labeled peptides determined as described in Section 2

Peptide	Amino acid sequence
1	M-E-G-I-ABDCys-R-N-R-V-T-N-N-V-K
2	Y-V-P-G-M-D-V-L-P-S-H-ABDCys-W-I-S-E-M-V-V-Q-L-S-D-S-L-T-D-L-L-D-K
3	L-V-N-I-V-D-D-L-V-E-ABDCys-V-K
4	D-F-V-V-A-S-E-T-S-D-ABDCys-V-V-S-S-T-L-S-P-E-K
5	M-E-G-I-ABDCys-R-N-R-V-T-N-N-V-K
6	D-V-L-P-S-H-ABDCys-W-I-S-E-M-V-V-Q-L-S
7	D-L-V-E-ABDCys-V-K-E-N-S-S-K
8	D-ABDCys-V-V-S-S-T-L-S-P-E-K
9	ABDCys-L-E-Q-V-R-K
10	L-ABDCys-A-T-Y-K
11	S-P-A-P-P-A-ABDCys-D-L-R-V-L-S-K
12	L-L-R-D-S-H-V-L-H-S-R-L-S-Q-ABDCys-P-E-V-H-P-L-P-T-P-V-L-L-P-A-V-D-F-S-L-G-E-W-K
13	A-Q-D-I-L-G-A-V-T-L-L-L-E-G-V-M-A-A-R-G-Q-L-G-P-T-ABDCys-L-S-L-L-G-Q-L-S-G-Q-V-R-L-L-L-G-A-L-Q-S-L-L-G-T-Q-L-P-P-Q-G-R-T-T-A-H-K
14	V-R-F-L-M-L-V-G-G-S-T-L-V-ABDCys-V-R-R-A-P-P-T-T-A-V-P-S

carried out for 7 h at room temperature. For the AspN peptide mapping, 200 μg of protein was diluted to 200 μl with 10 m*M* sodium phosphate, pH 7.0, containing 150 m*M* NaCl, 2 μg of AspN (an enzyme to protein ratio of 1:50) was added and the reaction was allowed to react at 37°C for 15 h.

Following the hydrolysis with either enzyme, the disulfide bonds were simultaneously reduced with TBP and alkylated with ABD-F according to [7]. The reaction mixture was incubated at 60°C for 10 min, and then cooled to room temperature. Immediately after reduction and alkylation, the peptides were acidified and injected directly onto a Vydac (Hesperia, CA, USA) 250×4.6 mm C_4 column equilibrated with buffer A [HPLC-grade water containing 0.1% trifluoracetic acid (TFA)]. The peptides were eluted with a linear gradient of 3–52% solvent B (90% CH_3CN, 10% HPLC-grade water and 0.1% TFA) over 90 min at a flow-rate of 0.7 ml/min. The gradient was held at 52% solvent B for 5 min, and then raised to 99% solvent B over 15 min (total run time was 115 min). All peptides were collected using the same gradient. Elution was monitored by absorbance at 215 nm and fluorescence (excitation at 385 nm and emission at 520 nm). Peptides containing ABD-labeled cysteines were collected from the fluorometer response and their sequences identified by N-terminal sequencing, matrix-assisted laser desorption (MALDI) MS and/or amino acid analysis. Peptide molecular masses before and after delabeling were determined by ESI-MS.

2.3. Delabeling of the cysteine containing peptides

Approximately 250–350 μg (as estimated by N-terminal sequencing) of each ABD–cysteine containing peptide was collected from the fluorometer response following reversed-phase chromatography and dried in a Speed Vac (Savant Instruments, Holbrook, NY, USA). After drying, the peptides were redissolved in 300 μl of 8 *M* urea and sonicated for 5 min. The peptide (25 μl) was diluted with 100 μl of 100 m*M* tricine (50 m*M* final), pH 7.0, 15 μl of 400 m*M* DTT (30 m*M* final) and the total volume was adjusted to 200 μl with distilled water and heated at 60°C for 0, 2, 4, 6, 8, 10, 15, 20, 25, 40, 60 and 70 min, respectively. After heating, the samples were cooled to room temperature, analyzed and quantitated by reversed-phase high-performance liquid chromatography (HPLC) using a Vydac 250×4.6 mm C_4 column. Two, 60 min gradients were used to analyze the treated peptides. All ABD-labeled peptides which eluted in 50% or less of solvent B (same as above) were analyzed using a 60 min linear gradient of 5–50% solvent B. All ABD-labeled peptides which eluted in greater than 50% solvent B were analyzed using a 60 min linear gradient of 30–99% solvent B. All peptide elutions were performed at 0.7 ml/min and monitored both for absorbance at 215 nm and fluorescence (same excitation and emission as above). The ratio between the peak area (from the absorbance at 215 nm) for the time zero sample versus the corresponding peak in each DTT treated sample was reported as the percent of label remaining. For each peptide, a single time treatment with DTT (as well as time zero) was chosen and the peaks contained in the chromatogram were analyzed for their component molecular mass. Peptide recoveries were determined to be greater than 85% (as estimated by N-terminal sequencing) for selected time points in the delabeling of peptide 10 (from Table 1). The estimated recoveries were similar to those seen previously in the delabeling of BSA [7].

2.4. Relabeling of delabeled peptides

Approximately 100 μg (as determined by amino acid analysis) of peptide 10 (see Table 1) was generated as previously described (see Section 2.2). The collected peptide was dried and reconstituted in 100 μl of 8 *M* urea. The peptide (25 μl) was diluted to 200 μl with tricine buffer at pH 8.0, injected immediately onto a Vydac 250×4.6 mm C_4 column and eluted with a linear gradient of 3–30% solvent B over 60 min. Elution was monitored by absorbance and fluorescence. The remainder of the peptide (75 μl) was delabeled using tricine buffer pH 8.0 containing 30 m*M* DTT. The delabeled peptide was collected, dried, reconstituted with 60 μl of 8 *M* urea and 25 μl was diluted to 200 μl with the pH 8.0 tricine buffer and injected directly onto the Vydac 250×4.6 mm C_4 column to obtain the chromatographic profile for the fully delabeled peptide 10. The remainder of the delabeled peptide was diluted to 200 μl with tricine buffer and relabeled with 2

m*M* ABD-F, heated for 10 min at 60°C, cooled and injected onto the reversed-phase HPLC column and eluted as above. This profile represents the relabeling of the delabeled peptide in the absence of reductant.

2.5. MALDI-MS

MALDI-MS was performed with a Kompact MALDI III mass spectrometer (Kratos Analytical, Ramsey, NJ, USA) fitted with a standard 337 nm nitrogen laser. The spectra were recorded with the analyzer in the linear mode at an accelerating voltage of 20 kV. MALDI-MS was used to determine the initial molecular mass of the purified ABD-labeled peptides. LC–MS analysis of the untreated or DTT treated ABD-labeled peptides were achieved on a HP1090 liquid chromatograph (Hewlett-Packard, Wilmington, DE, USA) using a Vydac 250×2.1 mm narrowbore C_4 column run at a flow-rate of 0.15 ml/min. The Perkin-Elmer Sciex API 100 mass spectrometer (Thornhill, Ontario, Canada) equipped with a nebulizer assisted ionspray source and operated in the positive ion mode was utilized for obtaining molecular mass data. The HPLC chromatograph was coupled to the mass spectrometer where the post-column line was split to reduce flow into the electrospray interface to 50 μl/min. The ionspray voltage was set at 4800 V, the orifice voltage was set at 50 V and the ring voltage was 400 V. All peptides were analyzed under the same voltage conditions.

3. Results and discussion

Previous work, which utilized BSA, was the first to demonstrate that the fluorescent ABD–cysteine label was reversible [7]. Upon extended treatment with a reducing agent 80–85% of the ABD label was found to be reversible. A similar degree of reversibility (78–91%) was observed for the parent proteins (data not shown) of the peptides investigated in the present study. It has been suggested [7] that the difficulty for the complete reversibility of the ABD label may be due to steric hindrance of cysteine residues when dealing with an intact protein. Therefore, in this work, we were interested in determining if peptides which contained an ABD–cysteine and varied in both length and amino acid composition were as difficult to delabel as was previously seen for BSA [7]. These purified ABD–cysteine containing peptides could further be used to define the structure of the cysteine residue upon removal of the ABD label.

Peptides which contain only a single cysteine residue can be generated from most proteins under enzymatic conditions. These purified peptides can be used to determine the reversibility at individual cysteine residues for the ABD adduct formed in the labeling reaction. The enzymes LysC and AspN generated peptides which contained only a single ABD–cysteine. All of the ABD-labeled cysteines were isolated for two of the three proteins utilized in the present study. In addition, the generated ABD–cysteine containing peptides varied in both the types of aliphatic versus hydrophilic residues contained and in their total amino acid content. After enzymatic hydrolysis, peptides were purified by reversed-phase chromatography and their sequences identified. These results are presented in Table 1.

Each purified ABD–cysteine containing peptide was initially delabeled for 10 min at 60°C in pH 7.0 buffer (tricine) containing 30 m*M* DTT. After heating, the peptides were analyzed by HPLC and compared to the chromatogram at time zero. Fig. 1 represents a typical reversed-phase chromatogram for the DTT treated peptides. These data indicate that two new peaks were generated (relative to the parent peptide) upon treatment. Both peaks were collected and their component peptides determined using N-terminal sequencing, MALDI-MS and amino acid analysis (when necessary). For every treated peptide, the non-fluorescent peptide was equivalent in amino acid content as the parent peptide at time zero. In addition, for all treatments, no sequence was identified in the 24 min fluorescent peak (see Fig. 1, panel B).

ESI-MS was used to determine the molecular mass of all peptides contained in the reversed-phase chromatograms before and after treating with DTT. Each peptide was treated for the same time interval (10 min) under constant conditions of temperature (60°C), DTT concentration (30 m*M*) and buffer (tricine, pH 7.0). The peptides at time zero were run under the same conditions as above minus the DTT. Only two gradients were used in the analysis of both the treated and time zero ABD-peptides (see Section

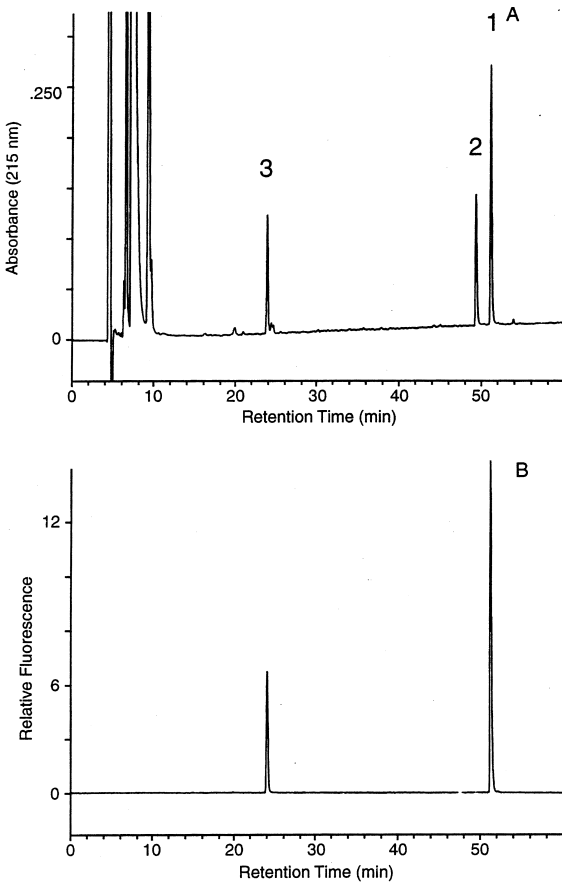

Fig. 1. Reversed-phase HPLC chromatogram of an ABD–peptide treated with DTT. Panel A: the response at 215 nm for peptide 7 (see Table 1 for amino acid sequence) after treating for 10 min at 60°C in pH 7.0 tricine buffer containing 30 m*M* DTT. ESI-MS data has identified the peak labeled as No. 1 to be the parent peptide containing the ABD label, peak No. 2 as the parent peptide minus the ABD label and peak No. 3 as an ABD–DTT adduct. Panel B: the fluorescence response for the peaks generated in panel A.

2.3). The ESI-MS data is presented in Table 2. These results indicate that, for all peptides, the molecular mass is consistent with the non-fluorescent peak being the parent peptide less the ABD label. In addition, the ESI-MS results are consistent with the regeneration of a free sulfhydryl upon the removal of the ABD label. Further, analysis of the ESI-MS data for the fluorescent peak (at 24 min in Fig. 1, panel B) generated upon treatment with DTT is consistent with the formation of an ABD–DTT adduct (observed ESI-MS molecular mass, M_r was 352). This was also shown by reacting only ABD-F with DTT. The resulting fluorescent peak had the same retention time and ESI-MS mass as the fluorescent peak generated in Fig. 1, panel B (data not shown).

With the identification of the component peaks, the degree of reversibility for the ABD label for these peptides was investigated as a function of time at a fixed DTT concentration. The delabeling conditions are described in Section 2.3. Fig. 2 represents the reversed-phase chromatograms for peptide 7, from Table 1, as a function of time of treatment in the presence of DTT. These chromatograms indicate that the non-fluorescent peak at approximately 49 min increases with time and was previously shown to be the parent peptide less the ABD label. The amount of ABD label remaining can be calculated from the peak area of the parent peptide remaining versus the peak area at zero time. These data are presented in Table 3 as the percent of ABD label remaining for the time indicated for each of the ABD-labeled peptides analyzed. These data are consistent with those previously shown for BSA [7] which indicate a decrease in ABD label remaining with time of incubation with DTT (see Table 3). However, unlike BSA [7], all of the ABD label could be completely removed from these peptides upon treatment with DTT.

The effect of pH on the reversibility of the ABD–cysteine was investigated for several ABD–cysteine containing peptides utilizing buffers made from tricine at pH values from 7.0–9.0. Like BSA [7], the rate of delabeling for the ABD–peptides increased as the pH of the reaction increased. Peptides 9 and 10 were examined for the change in rate of delabeling as a function of increasing pH. In both cases, increasing the pH to 8.0 resulted in approximately 90% removal of the ABD label after 6 min of treatment at 60°C in 30 m*M* DTT and 100% removal under the same conditions at pH 9.0.

The species generated by reversing the ABD-labeling of cysteine was shown to be a free sulfhydryl by delabeling peptide 10 (see Table 1), recollecting the delabeled peak and then relabeling with ABD-F in the absence of reductant (see Fig. 3). Panel A, represents the fully ABD-labeled peptide 10; panel B, represents the completely delabeled peptide 10 which was collected after treating with DTT; panel C, represents the delabeled peptide

Table 2
ESI-MS data: molecular mass data before and after removal of the ABD label with DTT for each of the cysteine containing peptides

Peptide (Table 1)	ABD-F	ESI-MS	
		Observed M_r	Expected M_r
1	+	1832	1832
1	−	1635	1635
2	+	3690	3690
2	−	3493	3493
3	+	1657	1657
3	−	1461	1657
4	+	2398	2398
4	−	2200	2201
5	+	1832	1832
5	−	1635	1635
6	+	2142	2142
6	−	1945	1944
7	+	1663	1664
7	−	1466	1467
8	+	1462	1463
8	−	1265	1265
9	+	1073	1073
9	−	896	896
10	+	876	876
10	−	700	699
11	+	1653	1652
11	−	1456	1455
12	+	4486	4486
12	−	4290	4289
13	+	6633	6632
13	−	6433	6433
14	+	2826	2827
14	−	2630	2630

(collected in panel B) which has been relabeled with ABD-F in the absence of reductant. Panel C of Fig. 3 demonstrates that the free sulfhydryl is generated upon delabeling since the ABD–cysteine can be regenerated in the absence of reducing agent (DTT). These results are consistent with the proven specificity of ABD-F for reaction with free sulfhydryls [12].

4. Conclusions

The increase in the rate of delabeling as a function of increasing pH reflects the increase in reduction potential of DTT [13]. The complete delabeling demonstrated for the ABD–peptides as compared to an intact protein suggest that the accessibility of the reductant to the ABD–cysteines, as originally suggested and which is probably due to incomplete denaturation under the conditions employed, is an important consideration when dealing with an intact protein. In addition, the primary amino acid sequence does not appear to effect delabeling further supporting the contention that steric hindrence prevented the complete delabeling of BSA. The current work involving purified ABD-labeled peptides illustrate

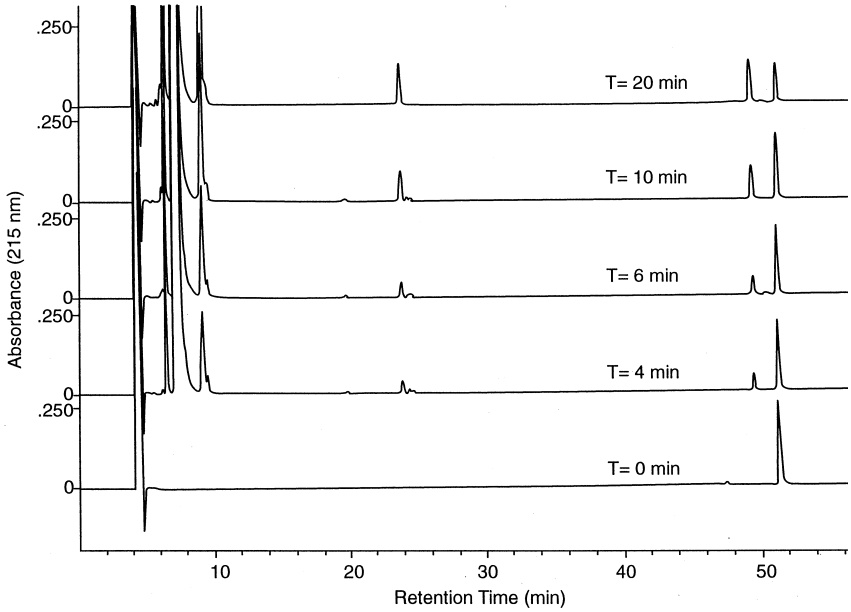

Fig. 2. Reversed-phase HPLC chromatograms representing the shift in retention time for the delabeled peptide as a function of time at 60°C. Peptide 7 (see Table 1 for amino acid sequence) was treated with 30 m*M* DTT, 50 m*M* tricine, pH 7.0, at 60°C for the time indicated.

that under the right conditions ABD–cysteines can be completely delabeled.

The ABD-labeled peptides have been used to directly illustrate that the structure of the cysteine, after removal of the ABD label, is a free sulfhydryl capable of reacting with any other alkylating agent. These results continue to support that ABD-F is an useful reagent for fluorescently labeling cyst(e)ine(s)

Table 3
Quantitation of the percent ABD label remaining for each of the purified cysteine containing peptides as a function of time at 60°C

Peptide	Time (min)											
	0	2	4	6	8	10	15	20	25	40	60	70
1	100	44	20	11	6	3	2	0	0	0	0	0
2	100	83	80	58	54	53	41	33	29	12	0	0
3	100	99	91	83	77	67	57	39	37	25	10	0
4	100	92	87	84	80	76	75	70	60	35	11	0
5	100	51	27	12	8	4	2	0	0	0	0	0
6	100	88	–	67	58	53	39	30	22	7	0	0
7	100	95	–	76	68	55	54	37	31	17	6	0
8	100	76	–	49	40	34	25	13	3	0	0	0
9	100	72	53	40	32	22	13	7	4	0	0	0
10	100	69	49	35	24	17	11	7	3	0	0	0
11	100	54	43	39	29	27	10	8	4	0	0	0
12	100	92	79	75	59	57	32	27	15	0	0	0
13	100	92	71	58	54	48	48	38	27	0	0	0
14	100	51	27	13	8	5	2	0	0	0	0	0

These data were generated as a function of time of incubation under the constant conditions of peptide concentration, 30 m*M* DTT, 50 m*M* tricine buffer, pH 7.0 and 60°C. The percent ABD label remaining for each peptide analyzed was calculated from the peak area of the fluorescently labeled parent peptide as compared to the peak area at zero time.

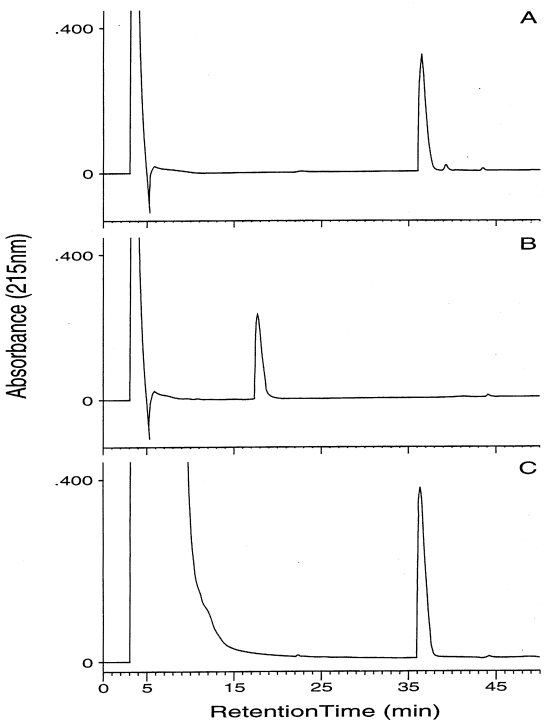

Fig. 3. Relabeling of a delabeled peptide in the absence of reductant. Panel A represents the fully ABD-labeled peptide 10 (see Table 1 for amino acid sequence); panel B represents the completely delabeled peptide 10 which was collected after treating with DTT; panel C represents the delabeled peptide 10 (collected in panel B) which has been relabeled with ABD-F in the absence of reductant.

with the added advantage of being completely reversible for further modification or analysis.

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