Published in final edited form as:

J Chromatogr A. 2008 June 20; 1194(2): 243–248.

Reaction of fluorogenic reagents with proteins I. Mass spectrometric characterization of the reaction with 3-(2-furoyl)quinoline-2-carboxaldehyde, Chromeo P465, and Chromeo P503

Roza Wojcik, Kristian E. Swearingen, Jane A. Dickerson, Emily H. Turner, Lauren M. Ramsay, and Norman J. Dovichi*

Department of Chemistry, University of Washington, Box 351700, Seattle, WA 98195-1700, USA

Abstract

3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ), Chromeo P465, and Chromeo P503 are weakly fluorescent reagents that react with primary amines to produce fluorescent products. We studied the reaction of these reagents with α -lactalbumin by mass spectrometry. The reaction generated a set of products by the addition of one or more labels to the protein. At room temperature, the reaction was an order of magnitude faster with the Chromeo reagents than with FQ; however, the steady-state labeling efficiency was a factor of two higher for FQ compared with the Chromeo reagents. The relative abundance of the products with FQ usually followed a binomial distribution, which suggests that the labeling sites were uniformly accessible to this reagent. In contrast, the distribution of reaction products with the Chromeo reagents did not follow a binomial distribution for reactions performed in the absence of sodium dodecyl sulfate (SDS); it appears that the protein labeled with the Chromeo reagents refolded into a relatively stable secondary structure that hid some reactive sites. The reaction with the Chromeo reagent did follow the binomial distribution if the protein underwent treatment with 1% SDS at 95 °C for 5 min, which apparently disrupts the protein's secondary structure and allowed uniform access to all labeling sites. Chromeo 503 labeled seven of 17 the 13 primary amines in denatured α -lactalbumin.

Keywords

Labeling chemistry; 3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ); Chromeo P465; Chromeo P503; Protein labeling

1. Introduction

Laser induced fluorescence is a particularly powerful tool for the characterization of minute amounts of proteins and other biogenic amines [1–3]. Unlabeled proteins can be detected based on native fluorescence, usually from tryptophan residues [4–6]. However, tryptophan is a relatively rare amino acid in many organisms [7]. Also, native fluorescence usually requires excitation with expensive and temperamental lasers that operate in the ultraviolet portion of the spectrum.

*Author to whom correspondence should be addressed: Department of Chemistry, University of Washington, Box 351700, Seattle, WA 98195-1700. Phone: (206) 543-7835. Fax: (206) 685-8665. E-mail: dovichi@chem.washington.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Non-covalent dyes have attracted attention for protein analysis in capillary electrophoresis [8,9]. These dyes are particularly attractive because they require minimal sample handling. Unfortunately, they suffer from several limitations. Some reagents generate relatively poor detection limits due to the large background signal from uncomplexed reagent, while others generate non-linear calibration curves. Also, charged complexing reagents are not compatible with isoelectric focusing.

A wide range of reagents has been developed to fluorescently derivatize proteins over the past 65 years [10–12]. These reagents usually couple a highly fluorescent dye, such as fluorescein, 6-[(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino]hexanoic acid (BODIPY), or cyanine dyes, with lysine or cysteine residues, and are particularly useful when labeling proteins as affinity reagents for immunoassay or immunostaining applications. In those experiments, the protein is labeled at high concentration and can be chromatographically purified and then diluted for use. However, highly fluorescent reagents are not particularly useful when analyzing proteins at very low concentrations. A large excess of reagent is usually required to drive the reaction to completion in a reasonable length of time. Unfortunately, trace fluorescent impurities present at the part-per-million level can obscure the signal generated by proteins present at low concentrations.

Fluorogenic reagents are attractive alternatives to conventional fluorescent dyes. Fluorogenic reagents are weakly fluorescent until they react with a primary amine to produce a highly fluorescent product. Novotny developed a set of fluorogenic reagents that react with primary amines in the presence of a nucleophile, such as cyanide, to produce isoindoles with excitation maxima in the visible portion of the spectrum [13,14]. We find 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) to be particularly useful for the ultrasensitive detection of proteins by capillary electrophoresis [15–18]. The products of this reagent with primary amines can be excited efficiently by relatively inexpensive and reliable 473 nm or 488 nm lasers, and labeled proteins have good electrophoretic properties in buffers containing anionic surfactants, such as sodium dodecyl sulfate (SDS) [15,17].

More recently, Wolfbeis has developed pyrylium compounds as fluorescent protein labels [19–21]. The reaction of pyrylium with a primary amine yields a positively charged pyridinium ion [22]. The single-step reaction proceeds relatively quickly at room temperature without need of a nucleophile. Two reagents are commercially available from Active Motif Chromeon as Chromeo P503 and P465; they generate products that can be excited at 473 or 488 nm. Although the company does not specify the structure of the compounds, their spectral characteristics are consistent with Py-1 and Py-5 reported by Wolfbeis [19].

We earlier employed mass spectrometry to monitor the reaction of FQ with ovalbumin [7]. That protein is heterogeneously glycosylated, which leads to a broad peak in its mass spectrum and which can obscure some of the details of the reaction. In this paper, we employ mass spectrometry to monitor the reaction of FQ, Chromeo P503, and Chromeo P465 with α -lactalbumin. This protein is not glycosylated and has relatively low molar mass (14 175 Da). The protein has 12 lysine residues plus an unblocked N-terminus, which provides 13 labeling sites.

2. Experimental

2.1 Chemicals

Chromeo P465 (Py-5) and Chromeo P503 (Py-1) were purchased from Active Motif Chromeon (Tegernheim, Germany). 3-(2-Furoyl)quinoline-2-carboxaldehyde and NaCN (FQ) were purchased from Molecular Probes (Eugene, OR, USA). Methanol 100.0% purity and acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Bovine α -lactalbumin,

≥85% purity, by polyacrylamide gel electrophoresis was purchased from US Biological (Swampscott, MA, USA). Sodium hydrogencarbonate 99.5% purity, SDS 99% purity, sodium tetraborate decahydrate 99.5–100.5% purity, trifluoroacetic acid, 99% purity, sinapic acid, 99.0% purity were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, 99.8% purity was purchased from EMD Chemicals (Gibbstown, NJ, USA). Potassium cyanide 99.9% purity was purchased from Fisher (Fair Lawn, N, USA).

2.2 Solutions

All aqueous solutions were prepared with deionized water from the NANO Pure Diamond system (Barnstead International, Dubuque, IA, USA). 100 mM stocks of sodium hydrogencarbonate, pH 9, sodium borate, pH 9 and SDS were filtered through a 0.22 µm filter (Millipore, Bedford, MA, USA) and diluted prior to use. Chromeo P465, P503, and FQ were dissolved in methanol and 100 nmol aliquots of the dyes were dried under vacuum using a Speed Vac (Savant Instruments, Farmingdale, NY, USA). Lyophilized aliquots were stored at –20 °C and reconstituted in methanol prior to use. A 2 mg/mL α-lactalbumin solution in deionized water was prepared fresh before each experiment. 10 mg/ml sinapic acid matrix-assisted laser desorption ionization (MALDI) matrix solution in 50% acetonitrile, 0.1% trifluoroacetic acid was prepared fresh, prior to use.

2.3 Labeling

Except for the denaturation study, labeling reactions were performed by mixing equal volumes of 50 µM α-lactalbumin solution in 20 mM buffer (borate or hydrogencarbonate, pH 9) and 1 mM dye solution in methanol. Aliquots were withdrawn from the reaction solution at the specified times and immediately mixed with ice cold matrix solution in at least 1:2 volumetric ratio and then placed on ice to ensure inhibition of labeling reaction.

For the experiments using a denatured protein, 1 mg/mL α-lactalbumin solution in 1% SDS was heated at 95 °C for 5 min. The solution was cooled and labeled using Chromeo P503. After labeling, SDS was removed from the denatured protein samples with Zeba Micro Desalt spin columns (Thermo Scientific, Rockford, IL, USA). Samples were then spotted on a stainless steel MALDI plate (Applied Biosystems, Foster City, CA, USA).

2.4 MALDI spotting and analysis.

A 0.5-µL volume of 10 mg/mL sinapic acid matrix solution was spotted on the MALDI plate and allowed to air dry. Then 5 pmol (0.5 µL) of sample previously diluted in matrix solution was spotted on top of the matrix and allowed to air dry. Samples were analyzed on an Applied Biosystems Proteome 4700 MALDI time-of-flight mass spectrometer equipped with 200 Hz Nd:YAG laser. Samples were analyzed in positive, linear acquisition mode. Typically, 3000 shots were accumulated for each spectrum. The mass axis was calibrated based on the known molar mass of α-lactalbumin.

2.5 Data analysis

Data were analyzed using Matlab running on a Macintosh computer. Data were treated with a 35-point, third-order Savitsky-Golay filter. The original data had a non-uniform spacing along the mass axis; the filtered data were resampled at every AMU. Next, a simple baseline correction algorithm was applied. Finally, a nonlinear regression routine based on the Marquart algorithm was applied to fit the data with a hypothetical mass spectrum formed by summing the mass spectrum of the unlabeled protein with the spectrum expected based on the addition of one to 13 labels to the protein.

3. Results and discussion

3.1 Room temperature kinetics

Figure 1 presents the mass spectrum of bovine α -lactalbumin. The spectrum consists of a main peak at 14,175 Da and a secondary peak at a few hundred Dalton higher molar mass. This second component is commonly observed in commercial preparations of this protein [23].

A 50 μ M solution of α -lactalbumin was allowed to react with a 1 mM solution of labeling reagent in a 10-mM borate buffer solution at room temperature. Figure 2 presents the mass spectra generated after 10 s, 1 min, 5 min, and 40 min of reaction. The smooth curves are the raw mass spectrum.

The set of peaks correspond to the spectrum of the protein in addition to the spectra of the protein with one or more labels, where each additional label increases the mass-to-charge ratio of the product by a constant amount, $\Delta m/z$. We performed a nonlinear regression analysis where the observed mass distribution was fit with the function

$$\text{intensity}(m/z) = \sum_{n=0}^{13} |a(n)| \times \text{native}(m/z + n \times \Delta m/z) \quad (1)$$

where $\text{intensity}(m/z)$ is the observed mass spectrum following labeling, $\text{native}(m/z)$ is the spectrum of the unlabeled protein, n is the number of labels added to the protein, $\Delta m/z$ is the mass increase following incorporation of a single label, and $a(n)$ is the probability of incorporating n labels; its absolute value is used to ensure that only positive contributions are considered. For α -lactalbumin, the maximum number of labels is 13. Fourteen parameters were used to fit the data [$a(0)$ through $a(14)$] in figure 2 and are shown as the dashed curve; the fits are essentially indistinguishable from the observed spectrum.

Figure 3 presents the distribution of the number of labels attached to the protein. There is a clear difference in the behavior of FQ and the Chromeo reagents. The Chromeo reaction is faster, approaching steady state within a few minutes at room temperature. In contrast, FQ reaction takes over 10 min to reach steady state at room temperature. However, the extent of reaction is greater for FQ than the Chromeo reagents, incorporating roughly twice as many labels at steady state.

There are 13 different labeling sites in α -lactalbumin. If each site is equally accessible to the labeling reagent, and if the incorporation of one label does not influence the labeling efficiency of subsequent labels, then the probability of labeling each site is a constant, p , and the distribution in the number of labels can be described by a binomial distribution:

$$P(n;m,p) = \frac{m!}{n!(m-n)!} p^n (1-p)^{m-n} \quad (2)$$

where $P(n;m,p)$ is the probability of observing n labels in a protein with m possible labeling sites; for α -lactalbumin, $m=13$. The mean number of labels attached is given by $\bar{n} = p \times m$. The distributions of figure 3 were fit with Eq. 2 and the results are shown as the smooth curves superimposed on the distributions; the mean number of labels attached is shown in the figure.

The distribution in the number of FQ labels attached is very well described by a binomial distribution. However, this agreement does not necessarily imply that all labeling sites are equally accessible; if all sites are equally accessible, then the reaction will go to completion, labeling all lysine residues. In contrast, the distributions observed for the Chromeo labels are not well described by a binomial distribution after 10 s reaction. The observed distributions show more unlabeled protein than predicted by the binomial distribution.

In all cases, the average number of labels follows first order kinetics,

$$\bar{n}(t) = n_{\max}(1 - e^{-t/\tau}) \quad (3)$$

where t is time, $n_{\max}$ is the number of labels observed at steady state, and τ is the lifetime of the reaction, figure 4. The reaction rate is an order of magnitude faster for the Chromeo reagents than for FQ, but roughly twice as many FQ labels are incorporated at steady state. This result is peculiar; if the Chromeo reagents are more reactive, we would expect that more reagent would be incorporated. However, the failure of the Chromeo reaction to follow a binomial reaction suggests that some labeling sites are much more accessible to these reagents, while the more hidden sites are not reactive during our experiment.

3.2 Reaction at elevated temperature

If the reactions of the Chromeo reagents are dominated by unfolding of the protein, then the reaction should be accelerated at elevated temperature. We investigated the reaction of Chromeo 465 at 50 and 85 °C. The mass spectra were fit with Eq. 1 (data not shown), and the resulting fits were used to describe the product distributions, figure 5. The reaction appears to be essentially complete after 1 min at both 50 ° and 85 °C, and the extent of reaction is essentially the same at the two temperatures. Roughly three times as many labels are bound at elevated temperature compared with the room temperature reaction, figure 3. The observed distributions of the number of attached labels are broader than predicted by a binomial distribution, shown as the curves in figure 5.

3.3 Effect of denaturation in the presence of SDS

The failure of the reagent incorporation to follow the binomial distribution implies that not all labeling sites are equally accessible to the Chromeo reagents under normal reaction conditions. The labeling reaction converts cationic lysine residues into a cationic product, which may allow the labeled protein to fold into approximately the same conformation as the unlabeled protein, shielding some sites.

To disrupt the protein's secondary structure, we treated it with 1% SDS at 95 °C for 5 min. Following treatment, the sample was then labeled with Chromeo P503 at 50 °C. Figure 6 presents the distribution in the number of incorporated labels for the denatured protein and a control sample that was labeled in the same fashion, but without the denaturation step.

The results are quite striking. The untreated sample incorporated an average of two labels, and the distribution shows significant deviation from the binomial distribution. The treated sample incorporated an average of seven labels; the reagent reacted with over half of the possible labeling sites. More importantly, the observed distribution follows the binomial distribution, which implies that all labeling sites are equally accessible and that the attachment of one label does not change the reaction rate of other sites for the denatured protein.

4 Conclusions

It is extremely difficult to exhaustively label all lysine residues of a protein [24]. As shown in this paper, most labeling reactions produce a mixture of products with different number of labels attached to the protein. The most incorporation of label occurs when the protein is denatured by treatment with SDS at elevated temperature before labeling. Nevertheless, the reaction inevitably produces a mixture of labeled products.

This labeling reaction mixture can be quite complex; a protein with m labeling sites can produce $2^m - 1$ different labeled products [25]. Each of these products can have different electrophoretic

or chromatographic properties, and labeled proteins can produce complex electropherograms during separation [26–28].

Mass spectrometry is not sufficient to predict the behavior of the labeled compounds during separation. The following two manuscripts in this journal present the electrophoretic and spectroscopic behaviors of proteins labeled with the Chromeo reagents [29,30]. Chromeo P503 appears to have favorable properties that facilitate analysis of complex mixtures of proteins from cellular homogenates.

Acknowledgements

We acknowledge support from the National Institutes of Health (R01GM071666).

References

1. Weiss S. Science 1999;283:1676–1683. [PubMed: 10073925]
2. Cheng YF, Dovichi NJ. Science 1988;242:562–564. [PubMed: 3140381]
3. Xu C, Zipfel W, Shear JB, Williams RM, Webb WW. Proc. Nat. Acad. Sci. USA 1996;93:10763–10768. [PubMed: 8855254]
4. Lee TT, Yeung ES. Anal. Chem 1992;64:3045–3051. [PubMed: 1463223]
5. Swaile DF, Sepaniak MJ. J. Liq. Chromatogr 1991;14:869–893.
6. Timperman AT, Oldenburg KE, Sweedler JV. Anal Chem 1995;67:3421–3426. [PubMed: 8686891]
7. Pinto D, Arriaga EA, Schoenherr RM, Chou SS, Dovichi NJ. J. Chromatogr. B 2003;793:107–114.
8. Harvey MD, Bablekis V, Banks PR, Skinner CD. J Chromatogr B 2001;754:345–356.
9. Wu S, Lu JJ, Wang S, Peck KL, Li G, Liu S. Anal Chem 2007;79:7727–7733. [PubMed: 17874848]
10. Coons AH, Creech HJ, Jones RN, Berliner EY. J. Immunol 1942;45:159.
11. Karolin J, Johansson LBA, Strandberg L. J. Amer. Chem. Soc 1994;116:7801–7806.
12. Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, Bhalgat MK, Millard PJ, Mao F, Leung WY, Haugland RP. J. Histochem. Cytochem 1999;47:1179–1188. [PubMed: 10449539]
13. Beale SC, Savage JC, Wiesler D, Wiestock SM, Novotny M. Anal. Chem 1988;60:1765. [PubMed: 3232812]
14. Liu LP, Hsieh YZ, Wiesler D, Novotny M. Anal. Chem 1991;63:408–412. [PubMed: 2064006]
15. Pinto DM, Arriaga EA, Craig D, Angelova J, Sharma N, Ahmadzadeh H, Dovichi NJ, Boulet CA. Anal. Chem 1997;69:3015–3021.
16. Lee IH, Pinto D, Arriaga EA, Zhang ZR, Dovichi NJ. Anal. Chem 1998;70:4546–4548. [PubMed: 9823714]
17. Zhang ZR, Krylov S, Arriaga EA, Polakowski R, Dovichi NJ. Anal. Chem 2000;72:318–322. [PubMed: 10658325]
18. Kraly JR, Jones MR, Gomez DG, Dickerson JA, Harwood MM, Eggertson M, Paulson TG, Sanchez CA, Odze R, Feng ZD, Reid BJ, Dovichi NJ. Anal. Chem 2006;78:5977–5986. [PubMed: 16944874]
19. Hoefelschweiger BK, Duerkop A, Wolfbeis OS. Anal. Biochem 2005;344:122–129. [PubMed: 16043111]
20. Wetzl BK, Yarmoluk SM, Craig DB, Wolfbeis OS. Angew. Chem., Int. Ed. Engl 2004;43:5400–5402. [PubMed: 15468079]
21. Craig DB, Wetzl BK, Duerkop A, Wolfbeis OS. Electrophoresis 2005;26:2208–2213. [PubMed: 15880625]
22. Katritzky AR. Tetrahedron 1980;36:679–699.
23. Keough T, Takigiku R, Lacey MP, Purdon M. Anal Chem 1992;64:1594–1600. [PubMed: 1503226]
24. Liu H, Cho B-Y, Krull IS, Cohen SA. J. Chromatogr. A 2001;927:77. [PubMed: 11572400]
25. Zhao JY, Waldron KC, Miller J, Zhang JZ, Harke H, Dovichi NJ. J. Chromatogr 1992;608:239–242. [PubMed: 1430027]
26. Craig DB, Dovichi NJ. Anal. Chem 1998;70:2493–2494.

27. Richards DP, Stathakis C, Polakowski R, Ahmadzadeh H, Dovichi NJ. *J. Chromatogr. A* 1999;853:21–25. [PubMed: 10486708]
28. Stoyanov AV, Ahmadzadeh H, Krylov SN. *J. Chromatogr. B* 2002;780:283–287.
29. Swearingen KE, Dickerson JA, Turner EH, Ramsay LM, Wojcik R, Dovichi NJ. *J. Chromatogr. A*. 2008submitted for publication (Part II: JCA-08-86).
30. Turner EH, Dickerson JA, Ramsay LM, Wojcik R, Swearingen KE, Dovichi NJ. *J. Chromatogr. A*. 2008submitted for publication (Part III: JCA-08-87).

Figure 1.
Mass spectrum of unlabeled α -lactalbumin.

Figure 2.

Mass spectra of α -lactalbumin reacting with FQ, Chromeo P503, and Chromeo P465. Aliquots were taken after the specified reaction time and diluted to quench the reactions before analysis. Solid traces are the mass spectrum. The dashed traces are the results of a least-squares fit to the data using Eq. 1.

Figure 3.

Distribution in the number of labels bound to α -lactalbumin at different reaction times. The observed distributions are shown as the bar charts and are obtained from the regression analysis of Figure 2. The curves are the least squares fits of a binomial distribution to the observed distributions. $\bar{n}$ is the average number of labels attached.

Figure 4.

Average number of labels incorporated versus reaction time. The data, shown as crosses, are from figure 3 and the smooth curve is the least squares fit of the data with Eq. 3. The fitting parameters are shown.

Figure 5. Distribution in the number of Chromeo P465 labels bound to α -lactalbumin for the labeling reaction performed at elevated temperatures. The bar charts are the results from the experiments and the smooth curves are the results of regression analysis using Eq. 3.

Figure 6.

Distribution in the number of Chromeo P503 labels bound to α -lactalbumin after a 10-min reaction at 50 °C. The denatured sample had been heated to 95 °C in the presence of 1% (w/v) SDS and then cooled to room temperature before the reaction. The bar charts are the results from the experiments and the smooth curves are the results of regression analysis using Eq. 3.