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Heterogeneity of protein labeling with a fluorogenic reagent, 3-(2-furoyl)quinoline-2-carboxaldehyde

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Abstract

Fluorogenic reagents are used for protein labeling when high-sensitivity fluorescence detection is required. Similar to traditional labeling with activated fluorescent dyes, such as fluorescein isothiocyanate, a fluorogenic reaction is expected to change the physical–chemical properties of proteins. Knowledge of these changes may be essential for efficient separation and identification of labeled proteins. Here we studied the effect of labeling of myoglobin with a fluorogenic reagent on the acid–base properties of the protein. The fluorogenic reagent used was 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ). In slab-gel isoelectric focusing, we found that the labeling reaction generated at least six species with p*I* values lower than that of non-labeled myoglobin. These species can be identified as products of progressive labeling of myoglobin with one to six FQ molecules. The same series of FQ-labeled species were observed when the reaction products were analyzed by capillary zone electrophoresis. The comparison of experimental and theoretical p*I* values allowed us to elucidate the labeling pattern—the number of FQ molecules corresponding to each labeled product detected by isoelectric focusing.

Keywords: Proteins; 3-(2-furoyl)quinoline-2-carboxaldehyde

1. Introduction

Fluorescence detection of proteins is characterized by very high sensitivity. To facilitate such detection, a protein can be labeled with either a traditional fluorescence dye or a fluorogenic reagent. A fluorescent dye fluoresces in a free form while a fluorogenic reagent becomes fluorescent only upon reacting with a protein. Labeling of proteins with fluorescent dyes typically requires purification of the labeled protein from the non-reacted dye to reduce the background fluorescence. Such a cleanup step can often be impractical. The excess fluorogenic reagent, in contrast, has negligible fluorescence, allowing one to detect labeled proteins in the presence of the excess fluorogenic reagent and without an additional cleanup procedure.

Fluorogenic reagents have been widely used for labeling of proteins for capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection [1–9]. A fluorogenic reagent usually modifies the exposed lysine residues and the terminal amino group of the protein. The labeling reaction can affect the separation performance since it changes the physico–chemical properties of proteins such as size, hydrophobicity, and isoelectric point. These effects

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may be quite drastic, taking into account that labeling typically generates a heterogeneous mixture of products. Due to steric hindrance, only a fraction of lysine residues potentially accessible for labeling is usually modified. The total number of labeled lysine residues may depend on the order in which these residues are modified. As a result, a heterogeneous mixture of incompletely labeled products is generated upon labeling of a single protein [9]. Since the acid–base properties of a fluorogenic reagent usually differ from those of a lysine group, multiple labeled products are expected to have distinct p*I* values. A modification of even a single ionogenic amino acid can lead to a considerable p*I*-shift, while not significantly changing the molecular mass. The products with different p*I* values will have considerably different electrophoretic mobilities in zone electrophoresis or isoelectric focusing (IEF). Therefore, to develop an electrophoresis method for protein separation, it is important to understand the pattern of labeling and to know the p*I* values of the multiple labeled products.

The fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) has been introduced by Novotny et al. and is now marketed by Molecular Probes Inc. [10]. This reagent is often chosen for its extraordinarily long Stocks shift ($\lambda_{ex}<500$ nm, $\lambda_{em}>600$ nm). FQ has been used in off-column and on-column labeling of proteins in CE [3]. Considerable heterogeneity of labeled products has been observed in both capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF) [10]. Unfortunately, p*I* values of the labeled products could not be determined from cIEF experiments due to the lack of appropriate fluorescent p*I* markers for cIEF. These values could not be determined using slab-gel IEF either, due to the inability of resolving labeled products using slab gel IEF in the previous attempts [9]. The lack of information on p*I* values prevented the authors from understanding the pattern of labeling.

In this work, we used slab-gel IEF and CZE to study multiple products of myoglobin labeling with FQ. We experimentally determined p*I* values of labeled products and compared them with those calculated theoretically. This analysis allowed us to determine the number of FQ molecules corresponding to each labeled product detected in IEF.

2. Experimental

2.1. Reagents

FQ was obtained from Molecular Probes (Eugene, OR). Acrylamide, *N*,*N*′-methylenebisacrylamide (Bis), *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (TEMED), Repel-Silane, Pharmalyte carrier ampholytes and an isoelectric focusing calibration kit were purchased from Amersham Pharmacia Biotech, Inc. (Oakville, ON). Myoglobin from horse skeletal muscle was bought from Sigma–Aldrich (Oakville, ON). Other chemicals were also supplied by Sigma–Aldrich unless otherwise specified.

2.2. Methods

2.2.1. FQ-labeling reaction

The FQ-labeling procedure was a slightly modified version of that described earlier [10,11]. Briefly, 9 μl of 3×10^{-5} *M* protein solution in water and 1 μl of 20 m*M* NaCN solution in water were added to a vial containing 50 nmol of dry FQ and shaken with a ''Vortex''. The low solubility of FQ in aqueous solutions did not allow complete dissolving of 50 nmol of FQ in 10 μl of water; however the conditions used ensured that the labeling reaction was carried out in a saturated solution of FQ. This, in turn, was essential for maximizing the rate of the labeling reaction. The reaction was carried out either at 25 °C for 15 min or at 65 °C for 3 min and stopped by placing the reaction vial in melting ice. The solution of the reaction products was diluted with a specified buffer in order to achieve the desired pH value and/or sample concentration. It was then subjected to centrifugation at $10\times g$ for 2 min to remove possible precipitate.

2.2.2. Slab-gel isoelectric focusing

The Multiphor II electrophoretic unit and EPS 35 XL power supply (Amersham Pharmacia Biotech, Inc.) were used for slab-gel experiments. The gels (5%T and 4%C) were 0.5 mm thick and contained either 5% (v/v) glycerol or 7 *M* urea. Two pH gradient ranges, 3–10 and 5–8, were used. After IEF, the gels were developed with Coomassie Brilliant Blue R-350 (standard protocol supplied by

Amersham Pharmacia Biotech, Inc.) or G-250 [12,13].

2.2.3. Capillary zone electrophoresis

The laboratory-made capillary electrophoresis instrument with LIF detection has been described in detail elsewhere [14]. Briefly, FQ-labeled proteins were excited with a 488-nm line of an Ar-ion laser (Melles Griot, Nepean, ON). Separation was carried out in 20 μm I.D., 150 μm O.D., 35–40 cm long fused-silica capillaries (PolyMicro Technologies, Phoenix, AZ) at 500 V/cm. The running buffer was either 2.5 m*M* borax at pH 9.1 or 10 m*M* phosphate at pH 6.8. A 250-pl sample plug was injected onto the capillary by a 3 s ×7.8 kPa siphoning pulse. If the borax run buffer was used, then between the runs, the capillary was washed by the successive pumping of NaOH, water and the running buffer for 1, 2, and 1 min respectively under a pressure of 60 p.s.i. When we used the phosphate buffer, the capillary was washed in between the runs by pumping the run buffer for 5 min under a pressure of 430 kPa.

3. Results and discussion

3.1. Theoretical consideration

FQ preferentially targets primary amines of lysine residues and the terminal amino group of proteins. Horse muscle myoglobin contains 19 lysine residues [15]. The three-dimensional structure analysis demonstrates that although all 19 lysines are exposed, they are distributed unevenly on the surface of the protein. Five lysine residues are found adjacent to other lysine groups and most of the others are separated by one or two non-lysine amino acids. Only five lysine residues are separated from other lysines by more than two non-lysine amino acids. Due to this close proximity of lysines and the relatively large size of the FQ molecule, we can expect considerable interference between FQ-labeling reactions of most lysine residues on the same protein molecule. Therefore, we anticipate that it is unlikely that all 19 lysines will be labeled. Moreover, depending on the order in which the amino acids undergo the reaction, different labeling patterns can be generated, resulting in the heterogeneity of labeled products.

FQ-labeling of a lysine group of a protein causes a change in its p*I* value. Since FQ does not bring a new ionizable group, the loss of a primary amine due to FQ-labeling results in the loss of 1 unit of positive charge and subsequently the shift in the isoelectric point of the protein. Here we used a published procedure to calculate the p*I* values of FQ-labeled products of myoglobin for a different number of FQ molecules attached to the protein [16]. The details and limitations of the calculation procedure are described elsewhere [16,17].

Fig. 1 is a comparison between theoretical p*I* values (line 1) and experimentally determined p*I* values (line 2, data taken from Fig. 2) of horse muscle myoglobin with an increasing number of FQ-modified lysine residues. Fewer than 10 FQ molecules per protein were detected experimentally (see below); therefore, we limited our calculations to 10 FQ labels per molecule of myoglobin. The modification of lysine with FQ causes the loss of 1 unit of positive charge; therefore, the p*I*-shift resulting from labeling is negative. We only distinguish between the species with different total number of labeled lysine groups. The value of p*I*-shift per FQ molecule decreases with an increasing total number of FQ labels (see Fig. 1). This behavior can be

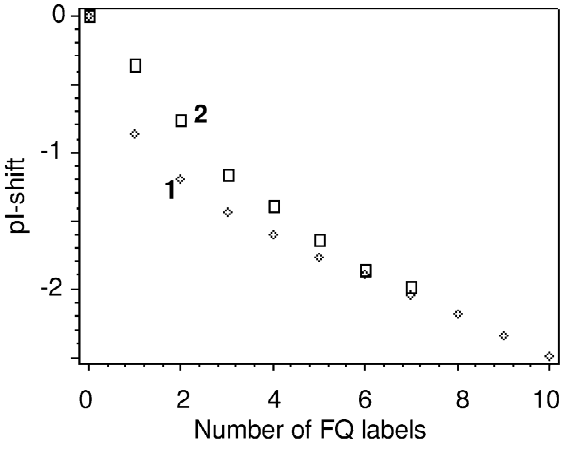

Fig. 1. The value of the p*I*-shift of myoglobin as a function of the number of FQ-labeled lysine groups: theoretical p*I* shift (line 1) and p*I*-shift determined experimentally with slab-gel IEF (line 2). The data for line 2 are obtained from slab-gel IEF depicted in Fig. 2.

Fig. 2. Slab-gel IEF of the intact (lanes 5–7) and FQ-labeled (lanes 1–4) myoglobin using Ampholites 3–10. The labeling reaction was carried out at 65 °C for 3 min for lanes 1, 2 and at 25 °C for 15 min for lanes 3, 4. Sample loading was performed from the anode for lanes 1, 3 and 7, from the cathode for lanes 2, 4, and 6, and from both sides for lane 5. The anode side is at the bottom.

explained by the absence of ionogenic groups with p*K* values in the neutral pH region [17].

3.2. Slab-gel IEF and CZE

Slab-gel IEF of non-labeled horse muscle myoglobin showed several isoforms with p*I* of the main isoforms being around 7.2 (Fig. 2, lanes 5–7). After FQ-labeling of the protein, at least six new bands appeared with a shift in the anodic direction (Fig. 2, lanes 1–4). The first of the new bands is separated from the band of non-labeled protein by approximately 0.5 pH units. The six bands occupy a total range of about 1.5 pH units. We ascribed the new bands to the FQ-labeled products of the main protein isoform. The minor myoglobin isoforms (Fig. 2, lanes 5–7) should also have multiple labeled products, but the limit of detection for the staining procedure used did not allow us to detect those products by IEF. Comparing theoretical p*I*-shifts with those determined experimentally allows us to understand the labeling pattern (see Fig. 1). The number of FQ molecules per protein varied from one to six.

The labeling pattern only changed slightly with temperature (compare lanes 1, 2 with 3, 4). When the reaction was carried out at 65 °C, the non-labeled protein was completely converted to the labeled products after 5 min. Further incubation did not change the relative amounts of the labeled products. Unexpectedly, the absolute amount of the products decreased with increasing incubation time. We explain this by the accumulation of another product(s) that precipitates in the place of sample application on the gel. This product could be observed in CZE as a broad band.

The CZE separation of FQ-labeled myoglobin at alkaline pH showed a set of peaks on the background of a broad band (Fig. 3A). By setting the pH value close to neutral, it was possible to achieve complete separation of the first six components from the band (Fig. 3B). We varied the incubation time in a range of 3 min to 1 h. The analysis of the reaction mixtures revealed a progressive decrease in the intensities of the six peaks and an increase in the intensity of the broad band with increasing incubation time.

In conclusion, in this work we demonstrate that acid–base properties of multiple labeled products of a protein with a fluorogenic reagent can be experimentally determined. We also showed that the information on p*I*-shifts caused by labeling can be used to understand the labeling pattern that is the number of FQ molecules per molecule of a protein. The understanding of the labeling pattern is essential for successful method development and analysis of labeled proteins by CE.

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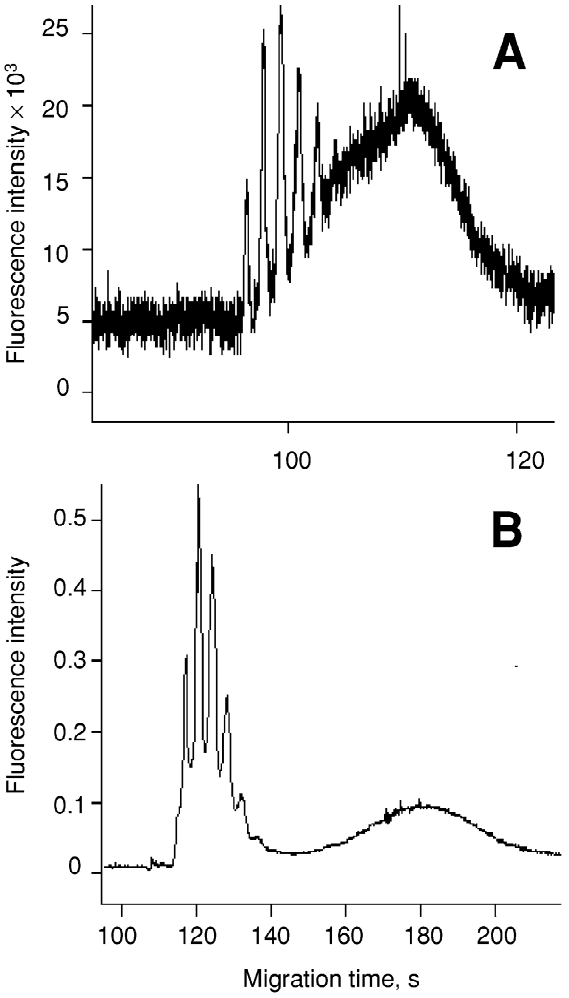

Fig. 3. CZE of FQ-labeled myoglobin in two running buffers: 2.5 m*M* borax at pH 9.1 (A) and 10 m*M* phosphate at pH 6.8 (B).

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