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Monitoring of a conjugation reaction between fluorescein isothiocyanate and myoglobin by capillary zone electrophoresis

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Abstract

The conjugation reaction between the amine reactive fluorescent probe fluorescein isothiocyanate and the protein horse heart myoglobin was directly monitored as the reaction progressed by capillary zone electrophoresis with UV absorbance detection. Kinetic analysis of the data indicated that the order of the reaction was first with respect to myoglobin and 1.3 with respect to fluorescein isothiocyanate. The separation of peaks attributable to the incorporation of $n = 1, 2, \ldots 7$ fluorochrome labels into the protein has some significance for the generation of single-label protein probes, such as antibodies.

1. Introduction

Capillary electrophoresis (CE) is a relatively new analytical technique which is presently experiencing a rapid growth as increasing numbers of researchers discover the power of the technique for the separation of a wide range of molecules. Recently, a comprehensive review of the technique has been published which chronicles developments made in the fundamental understanding, instrumentation, and applications of the technique [1]. One area that has attracted much interest is the CE separation of proteins. It was originally supposed that exceptionally sharp zones would be achieved for protein separations since they possess relatively small diffusion coefficients [2]. However, capillary wall interactions lead to zone broadening and sometimes, to irreversible adsorption. Numerous methods have been developed to correct this problem [3], although no one method ensures satisfactory performance for all proteins.

Other problems exist with CE separations. The narrow bore of the capillary puts severe limitations on detector technology. When used as a detector for CE, the ubiquitous absorbance detector must be made to work with a path length which is typically at least two orders of magnitude shorter than that for standard spectrophotometers using 1 cm path lengths. This corresponds to relatively high limits of detection (10^{-5}–10^{-6} M). Furthermore, the dynamic range of the technique will be very narrow as the high concentration limit is controlled by the ionic strength of the run buffer. Typically, if the concentration of the run buffer is not significantly greater than the analyte concentration (i.e. [run buffer] > 100[analyte]), localized po-

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tential gradients can be created leading to irregular peak shapes [3]. Therefore, for a 10 m*M* run buffer, the best possible dynamic range is 1 to 2 orders of magnitude. For this reason, other detection methods have been investigated which can lower the detection limit and thereby broaden the dynamic range for the technique.

Fluorescent methods are attractive for this purpose. Amine-reactive fluorescent probes can be used to create fluorescent derivatives of non-fluorescent analytes to allow for their sensitive detection. Cheng and Dovichi [4] have demonstrated sub-attomole (10^{-18} mol) mass detection limits for fluorescein isothiocyanate (FITC) derivatives of amino acids. This performance has subsequently been improved to the zeptomole range with picomolar (10^{-12} *M*) concentration detection limits [5]. This effectively drops the detection limit by approximately six orders of magnitude and increases the dynamic range of the technique to at least five orders of magnitude.

However, if the analyte of interest contains more than one reactive site, a complex mixture of products is typically obtained in a conjugation. This is due to the fact that the reactive fluorescent probes are quite bulky and the reaction process does not go to completion. Thus numerous products are produced differing in the number and spatial orientation of the conjugated probe [6]. It has been shown that for a molecule with n primary amino groups, $2^n - 1$ reaction products are possible [7]. The separation power of CE is capable of partially resolving this complex mixture leading to complex electropherograms with one analyte and indecipherable electropherograms with more than one. For the CE analysis of proteins, which contain numerous possible conjugation sites, this becomes catastrophic for practical analyses.

For amine-reactive fluorescent probes like FITC, conjugation to proteins occurs almost exclusively through nucleophilic attack by the ϵ-amino group of lysine residues and the N-terminal α-amino group [8]. Although the reaction is quite specific, proteins typically contain numerous lysine residues. Myoglobin, for example, contains 19 lysine residues and 1 N-terminal amino group[1]. Thus $2^{20} - 1 = 1\,048\,575$ different products are possible from a conjugation reaction. Somewhat fortunately, this formidable number is not fully realized, but one would still expect numerous products in a conjugation reaction with FITC.

In this study, we have monitored the conjugation reaction between myoglobin and FITC with time to obtain kinetic data and better understand the conjugation process. If the reaction proves to be second order overall, then FITC is incorporated into the protein in a stepwise fashion. Therefore, there should presumably be a point early in the conjugation reaction where a single, unique product with one FITC molecule incorporated is yielded. This would be of significant interest to CE separations that rely on fluorescence detection.

2. Experimental

2.1. Apparatus

CE separations were performed on a ATI-Unicam (Mississauga, Canada) Model Crystal 310 system with a 48-position peltier-cooled autosampler. Injection reproducibility of 0.40% R.S.D. based on peak area for 42 replicate dynamic compression injections is claimed for cytosine using this instrumentation [9]. In this study, dynamic compression injections of 10 mbar for 0.1 min were used. The sample tray in the Crystal 310 system could be temperature controlled from 4 to 40°C with a stability of ± 1° C. All conjugations were performed at 20 ± 1°C. Conjugation solutions were contained in glass vials sealed with Starburst caps to prevent evaporation, but allow periodic sampling by the Crystal 310 system. Detection was provided using a Unicam (Mississauga, Canada) 4225 UV-absorbance $^2H_2$ lamp-based detector operating at 200 nm. Absorbance signals where collected at 10 Hz using a National Instruments (Austin, TX, USA) NB-MIO-16X-H (16 bit resolution) data acquisi-

[1] Data from the Swiss Protein Data Base at the European Molecular Biology Labs.

tion board and stored as binary files on the hard disk of an Apple Computer (Cupertino, CA, USA) Model Macintosh Quadra 650 personal computer using an application developed in the National Instruments graphical programming language, LabView 3.0. The binary files were then read into the application program, Igor (WaveMetrics; Lake Oswego, OR, USA) where the data could be presented in graphical form.

2.2. Conjugations

Myoglobin was selected as the model protein for this study since excellent CE separation efficiencies have been demonstrated for this protein by simply using a run buffer with a pH that is significantly higher that the isoelectric point (p*I*) of the analyte. Lauer and McManigill [10] used this method to achieve theoretical plate counts of over 200 000 for both horse and sperm whale myoglobin. Myoglobin conjugations with FITC were achieved using molar ratios of 5:1 and 10:1, dye to protein. Typically, 5 mg/ml solutions of myoglobin in run buffer (0.1 *M* borate, pH 9.2) were used. Conjugation was initiated by adding an aliquot, which was 10% of the volume of the myoglobin solution, of FITC in N,N-dimethylformamide (DMF). The addition of the cosolvent DMF resulted in an increase in pH by about 0.3 pH units in the conjugation solution, which was deemed insufficient to effect the conjugation. Indeed, DMF is compatible with most aqueous protein solutions even at up to 30% (v/v) ratios [8].

2.3. Separations

All separations were performed with Polymicro (Phoenix, AZ, USA) 60 cm × 50 μm I.D. × 350 μm O.D. fused-silica capillaries. A small window was burned in the polyimide coating of the capillary 10 cm from the grounded outlet buffer vial. Capillaries were treated before use by flushing them with 0.1 *M* KOH for approximately 5 min, followed by HPLC-grade water, then run buffer, 0.1 *M* borate, pH 9.28, for similar durations. The capillary was then left in run buffer overnight. All separations followed a similar methodology that could be programmed into the Crystal 310 system. Initially, the outside of the capillary was rinsed by immersing it in a vial of run buffer for 0.01 min, then a sample was injected for 0.1 min, followed by another rinse in the same initial vial, then the capillary was immersed in a separate vial containing run buffer used for the separation. Separations were initiated by applying positive potential (24 kV) to the inlet buffer vial through a Pt–Ir (80/20) wire. Current flow in the capillary was approximately 44 μA. After separation, the capillary was purged using 2300 mbar of pressure for 0.5 min, making it ready for the next analysis.

2.4. Molar ratios of conjugated FITC to myoglobin

The molar ratio of conjugated FITC to myoglobin (F/P) was obtained by two methods. Both methods relied on stopping the conjugation reaction by the addition of at least a 50-fold molar excess of hydroxylamine (pH adjusted to 8.5 with 5 *M* NaOH) relative to the initial FITC concentration. The conjugation reaction solution was then passed through a Sephadex G-25 column equilibrated with run buffer to remove the hydroxylamine–FITC conjugate. In the first method, the electropherogram of the purified solution was obtained and areas of the individual peaks attributable to successive numbers of FITC conjugated to myoglobin were integrated using the application program, Igor. This peak area is due to the sum absorbance from myoglobin and the FITC labels, however. Therefore the relative extinction coefficient for FITC to myoglobin was determined at 200 nm using pure standard solutions of the two compounds. This was determined to be 0.022. The individual peak areas then had to be corrected for the contribution from FITC absorbance. For example, the peak area attributable to M + 1F was subtracted by 0.022(peak area), M + 2F by 2(0.022)(peak area) and so on. The average number of FITC labels incorporated into the myoglobin was then computed according to the equation:

$$\text{Average F/P} = \frac{\sum_{n=0}^{8} nM_n(1-c)}{\sum M_n} \quad (1)$$

where c refers to the relative extinction coefficient for FITC to myoglobin, n to the number of FITC peaks incorporated into myoglobin ($n = 0$–8) and M_n to the area of each peak attributable to a separated M + nF component.

In the second method, the absorbances of the purified conjugation reaction mixture were measured at 409 and 490 nm using an Hewlett-Packard (Palo Alto, CA, USA) Model 8451A diode array spectrophotometer. As the extinction coefficient (ϵ) of FITC is reduced approximately 10% on protein conjugation, the extinction coefficient for FITC incorporated into myoglobin was taken to be 68 000 $cm^{-1}\ M^{-1}$ [11]. The extinction coefficient for Fe(III) in the heme group of myoglobin which absorbs at 409 nm was taken to be 188 000 $cm^{-1}\ M^{-1}$ according to Tamura et al. [12] The F/P was then calculated according to the following equation:

$$\text{Average F/P} = \frac{A_F \epsilon_P}{A_P \epsilon_F} \quad (2)$$

Spectra obtained for a pure FITC solution in run buffer demonstrated insignificant levels of absorbance at 409 nm so that the absorbance measurement at 409 nm for the protein conjugate was assumed to be totally due to the heme group of myoglobin.

2.5. Chemicals

Myoglobin from horse heart (95–100%), Sigma-grade borax (approx. 99%) and Sephadex G-25 fine, were purchased from Sigma (St. Louis, MO, USA). HPLC-grade DMF was purchased from Aldrich (Milwaukee, WI, USA). Fluorescein-5-isothiocyanate, Isomer I (FITC) was purchased from Molecular Probes (Eugene, OR, USA). Hydroxylamine hydrochloride (99% purity) was obtained from ICN Biochemicals (St. Laurent, Canada).

3. Results

Fig. 1 contains an electropherogram of horse heart myoglobin at a concentration of 5 mg/ml. DMF has been added to the solution to conform to the reaction conditions used for conjugation. There are two well separated peaks for myoglobin which is expected from the reported dual isoelectric points [13]. Lauer and McManigill's technique separates according to pI, with the higher pI protein eluting first due to less associated negative charge [10], therefore the larger peak eluting first is due to that portion of myoglobin with a pI of 7.3. The smaller peak due to myoglobin (pI 6.8) is at a sufficiently low concentration (peak height is $>10\times$ smaller than that for pI 7.3) that it is not a detectable participant in the conjugation. The theoretical plate count for myoglobin (pI 7.3) is approximately 200 000 based on calculations using peak widths at half maximum.

Fig. 2 contains a number of electropherograms of conjugation reactions occurring between myoglobin and FITC at dye-to-protein molar ratio of 5:1. Each electropherogram serves as a snapshot of the reaction's progress. In examining the electropherograms, an obvious development as the time coordinate of the conjugation reaction increases is the appearance of an increasing number of peaks in the electropherograms while the myoglobin peak height decreases. These peaks are due to an increasing number of fluorescent probes being conjugated per protein molecule. As the fluorochrome is incorporated into the protein through lysine residues, a positive charge associated with the protein is replaced by a negative charge since at the pH of the conjugation/run buffer, the ϵ-amino group of lysine is positively charged ($pK_{R\ group}$ 10.5 for lysine amino acid [14]), whereas fluorescein is negatively charged. It is realized that the $pK_{R\ group}$ can be altered through conjugation to proteins and is dependent on the spatial environment of the residue in the protein's quaternary structure, but the pH of the run buffer is sufficiently lower than 10.5 that all lysine residues in the myoglobin are positively charged in the run

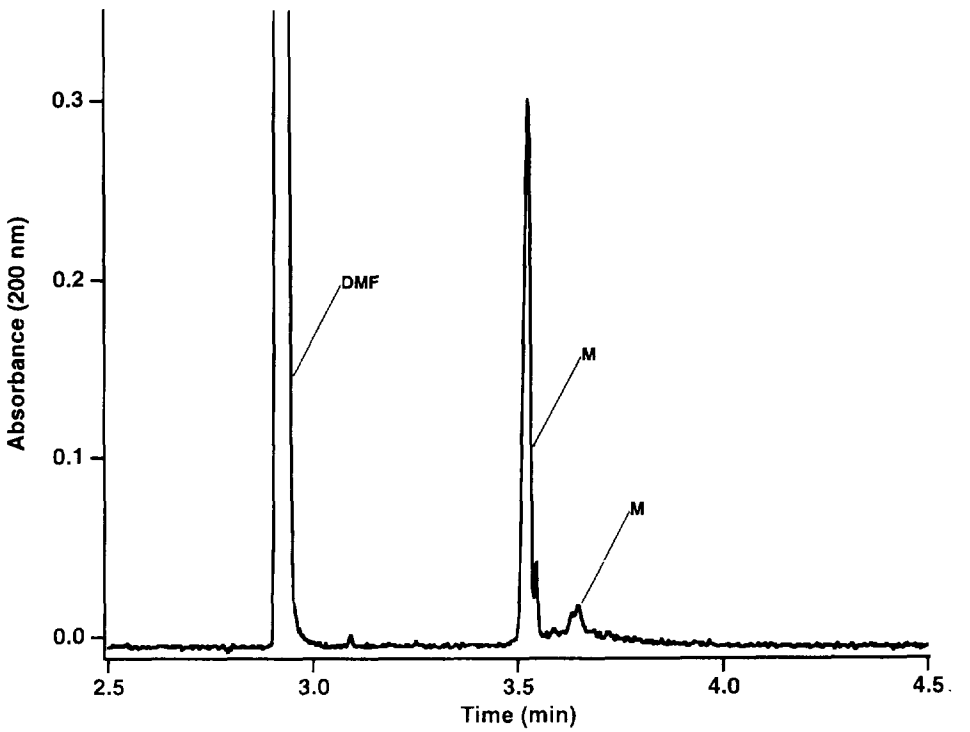
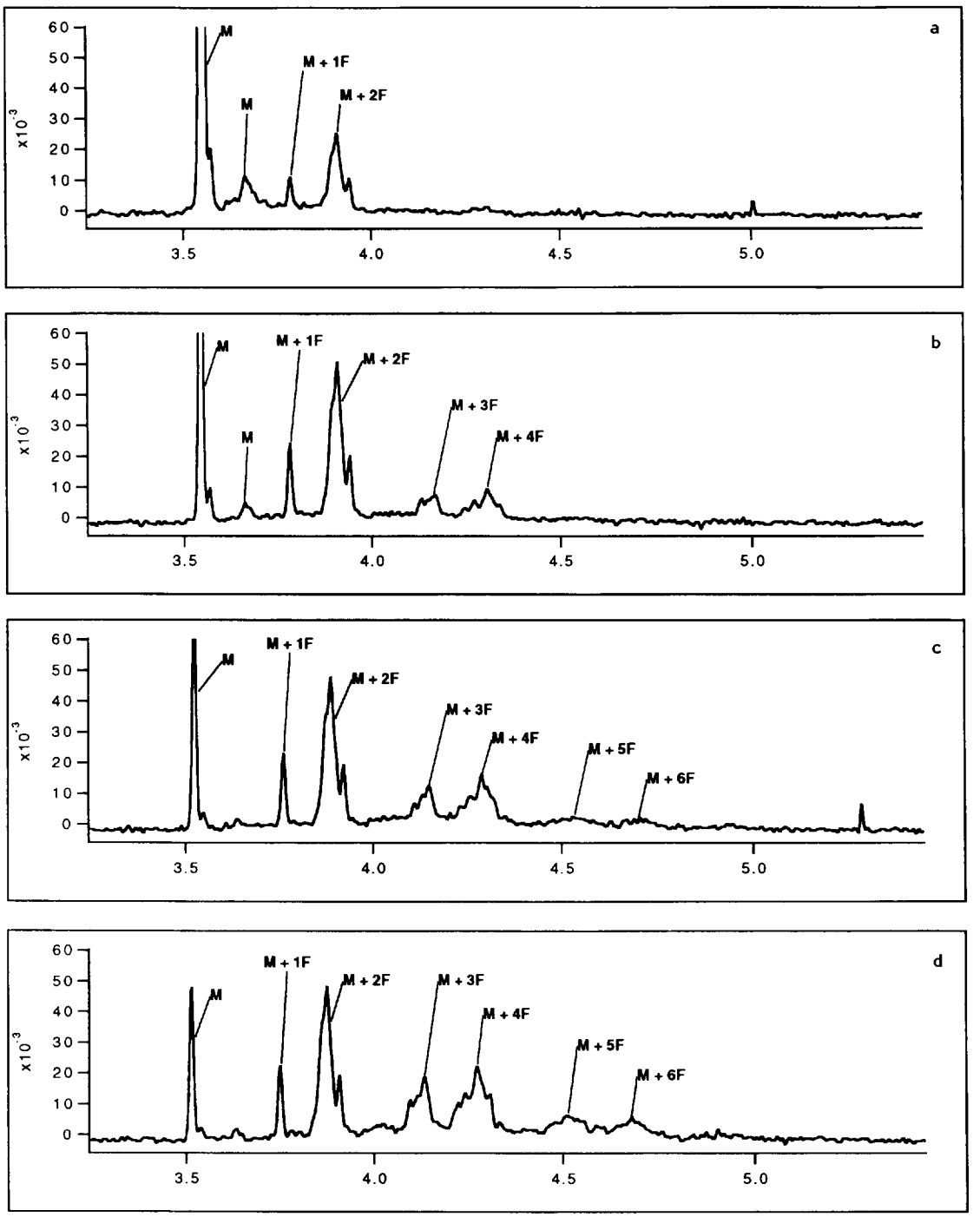


Fig. 1. Electropherogram of 5 mg/ml horse heart myoglobin in run buffer, 0.1 *M* borate, pH 9.28, with 10% DMF. Peaks labelled with an M are attributable to myoglobin.

buffer. Therefore, conjugation with a fluorescein reactive probe effectively changes the p*I* of the protein, which can be resolved from the pure protein. In all there are individual peaks in the electropherograms due to myoglobin being conjugated with from 1 to 7 FITC molecules. The relatively equal spacing between these peaks supports this hypothesis. Furthermore, myoglobin from horse heart muscle contains 19 lysine residues[2], therefore there are certainly enough conjugation sites for this degree of conjugation.

This hypothesis was verified by comparing the molar ratios of conjugated dye to protein estimated by the peak areas in the electropherograms and the more conventional spectrophotometric methods using absorbance measurements and extinction coefficients. Fig. 3 contains the electropherogram for a 10:1 FITC-to-myoglobin conjugation that was stopped by the addition of 50-fold excess of hydroxylamine relative to the initial FITC concentration after approximately 1 h of reaction time has elapsed. The mean of this distribution, computed using Eq. 1, equal to the average F/P, is 4.6. The F/P was spectrophotometrically determined to be 4.1 which demonstrates an acceptable agreement with that obtained from the electropherogram and tends to confirm the peak assignment used in Figs. 2 and 3. The spectrophotometric determination of F/P appears to be more accurate than that acquired from the peak integration method since the peak area of the FITC peak for a 10:1 conjugation reaction is approximately 40% smaller after 1 h conjugation reaction time compared to a blank. An improved performance would be obtained for the peak integration method if a Vis detector were available since absorbance measurements

[2] Data from the Swiss Protein Data Base at the European Molecular Biology Labs.


a
M
M
M + 1F
M + 2F
b
M
M
M + 1F
M + 2F
M + 3F
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c
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M + 2F
M + 3F
M + 4F
M + 5F
M + 6F
d
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M + 1F
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Fig. 2.

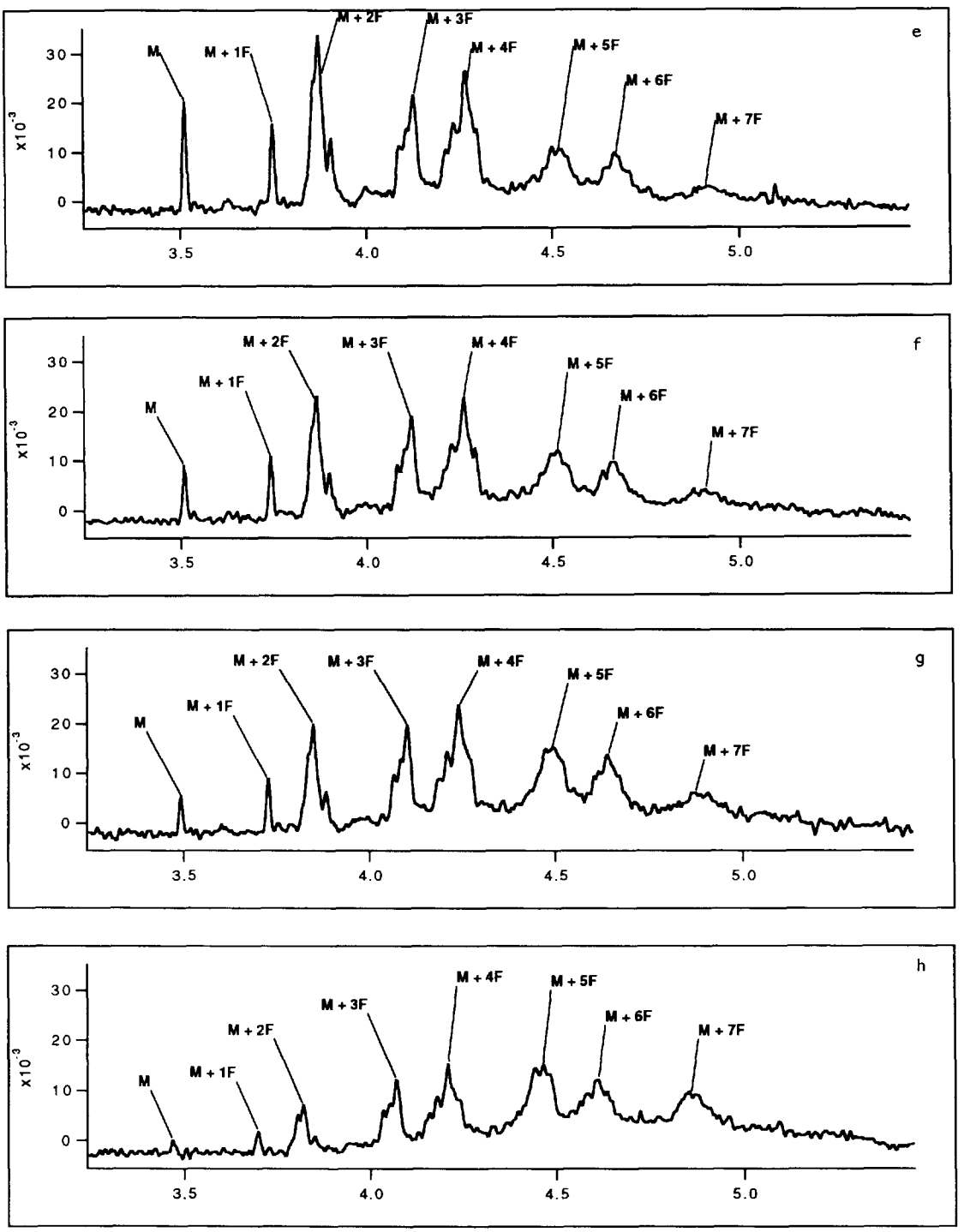

Fig. 2. Electropherograms for a 5:1 molar ratio conjugation of FITC to myoglobin at various times after the initiation of the conjugation reaction: (a) 2 min; (b) 19 min; (c) 36 min; (d) 52 min; (e) 85 min; (f) 110 min; (g) 135 min; (h) 218 min. Peaks labelled with M are attributable to myoglobin; M + 1F to myoglobin with one FITC molecule conjugated; M + 2F to myoglobin with two FITC molecules conjugated; . . . ; M + 7F to myoglobin with seven FITC molecules conjugated.

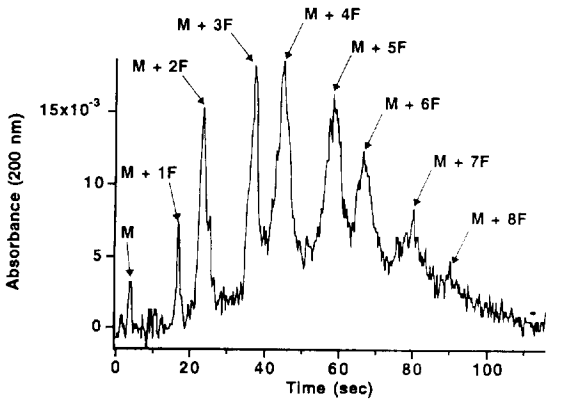

Fig. 3. Electropherogram for a 10:1 molar ratio conjugation of FITC to myoglobin which has been stopped by the addition of hydroxylamine after 1 h and the conjugation mixture passed through a G-25 column. Peak assignments as in Fig. 2.

at 409 nm would be directly attributable to the heme group of myoglobin with no significant interference from FITC.

Fig. 4 shows time profiles for some of the peaks evident in the electropherograms for the 5:1 molar ratio. Since peak height is directly proportional to concentration according to Beer's Law, kinetic analysis of the data can be made. The myoglobin peak follows an exponential decline described by pseudo-first order kinetics due to the excess concentration of FITC. An effective rate constant of 0.023 min^{-1} was obtained from the slope of a plot of the natural logarithm of the myoglobin peak height versus conjugation reaction time from 0 to 170 min. The straight line correlation for the plot was 0.996. The peak height of the myoglobin + 1 fluorochrome (M + 1F) peak experiences a doubling in peak height within 20 min of its first appearance. This is followed by a gradual decline in peak height throughout the remainder of the conjugation reaction. The apex of the M + 1F peak coincides with the time where the myoglobin's peak height is 1/e that of its initial height. This seems appropriate since the production of M + 1F should slow as the myoglobin is consumed and the M + 1F fraction reacts further with FITC to produce M + ($n > 1$)F peaks. The production of peaks with greater degrees of conjugation is evident by examining the M + 5F peak which slowly grows with increasing conjugation reaction time. However, approximately 40 min of reaction time is needed before the M + 5F peak is produced in detectable amounts.

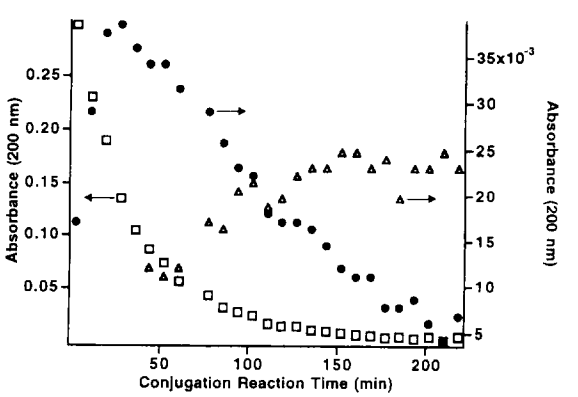

Fig. 4. Conjugation reaction time profiles for myoglobin, M, peak height absorbance (□); M + 1F peak height absorbance (●); and M + 5F peak height absorbance (△).

In general, from the analysis of the electropherograms, it is evident that the higher conjugated myoglobin fractions grow as the conjugation reaction proceeds at the expense of the lower conjugated myoglobin fractions. After nearly 4 h of reaction in the 5:1 molar ratio, no peak attributable to myoglobin is evident and little M + 1F or M + 2F. Even the peak heights of M + 3F and M + 4F have begun to diminish.

Another interesting aspect in the electropherograms is that lower conjugated protein peaks display sharper zones. Numerous peaks appear to be underlayed by the M + nF envelopes. Upon expansion of the peaks along the time axis, the M + 2F peak seems to be comprised of perhaps four individual peaks, M + 4F of five peaks and M + 7F of eight peaks. This indicates that separation is based not only on pI changes induced by the degree of conjugation, but also to the relative position of the dye labels in the protein conformation, albeit to a lesser extent. Also evident in the electropherograms is the development of a broad background with time, on which the peaks are superimposed. If the reaction is allowed to continue for 24 h, the broad background increases further and shifts to

longer migration times indicative of multi-conjugation of the protein.

If the ratio of FITC to myoglobin is increased to 10:1, little difference is seen except for an approximate tripling of the effective rate constant to 0.060 min^{-1} due to the doubling of the FITC concentration. The straight line correlation for this plot is 0.998, which is marginally better than that for the 5:1 ratio conjugation as one would expect as the pseudo-first order kinetics are more closely approximated. The broad background noted in the 5:1 conjugation ratio electropherograms appears earlier in the 10:1 ratio conjugation, as expected.

An interesting conclusion can be made from this kinetic analysis. The tripling of the effective rate constant with an increase in FITC concentration by two suggests that the overall order of the reaction is not second. The effective rate constant is related to the product of the true rate constant of the reaction and the concentration of the FITC to whatever order (α) it participates in the reaction (see Eq. 3).

$$k_{eff} = k[\text{FITC}]^{\alpha} \quad (3)$$

where k_{eff} is the effective rate constant, k is the true rate constant and [FITC] is the initial concentration of FITC, assumed to remain unchanged throughout the conjugation reaction. For α to equal 1 and the conjugation reaction be described by second-order kinetics, the doubling in concentration of FITC should result in a like doubling in the effective rate constant. However, k_{eff} appears to triple. If the initial FITC concentration in the conjugation reaction is tripled and quadrupled relative to the 5:1 conjugation, the effective rate constant was evaluated to be 0.11 and 0.14 min^{-1}, respectively. If the natural logarithm is taken of both sides of Eq. 3, α can be obtained from the slope of the straight line from a plot of ln k_{eff} versus ln [FITC]. This was found to be 1.3. Therefore the rate equation for the conjugation of FITC to myoglobin may be written:

$$-\frac{\text{d[myoglobin]}}{\text{d}t} = k[\text{myoglobin}][\text{FITC}]^{1.3} \quad (4)$$

This hypothesis could have been verified by fully applying the Ostwald isolation method (see [15]) and monitoring a conjugation reaction with a large excess of myoglobin relative to FITC to accurately determine if FITC is a first-order participant in the conjugation reaction. This was not practical with the current methodology, however, as large amounts of injected protein tended to influence the electrophoretic performance and yield irreproducible results.

4. Discussion

From the "snap shot" electropherograms of the myoglobin–FITC conjugation, it is evident that at no point in the conjugation is there a single, unique peak attributable to a fluorescent protein conjugate. After 2 min of conjugation for the 5:1 conjugation, there are two peaks, attributable to 1 and 2 FITC molecules conjugated to the protein. Furthermore, if areas are computed for these peaks, the M + 1F peak represents only 3% of the total protein concentration in the conjugation reaction mixture; the M + 2F peak only 18% in the 5:1 conjugation. Thus, only approximately 20% of the myoglobin has been conjugated. By the time these peaks have become more significant, M + 3F and M + 4F peaks have further complicated the electropherogram. In addition, the kinetic analysis indicates that FITC is not incorporated into myoglobin in a stepwise manner, therefore it is improbable that a single, unique peak can be obtained. An improved performance could possibly be obtained with other fluorescent reactive probes and will be the subject of further study in this research group.

On a brighter note, the complete separation of the M + 1F peak from both myoglobin and the M + 2F peak suggests a means for generating single-label antibody probes which may be used for analyses demonstrated by Shimura and Karger [16] group recently. Using rather complex chemical techniques, they were able to generate μg amounts of single-label antibody directed against methionyl recombinant human growth hormone, which were sufficient to perform several thousand assays.

In the 5:1 conjugation reaction after 19 min, the M + 1F peak represents approximately 7% of the total protein peak area. The injection plug is about 6 nl, so for the 5 mg/ml sample, the M + 1F peak represents 2 ng of protein, or enough for several assays if the protein in question was an antibody. So if a similar separation of the conjugation reaction could be made using an antibody as the protein, presumably one could collect a single-label product using fraction collection techniques.

Cheng et al. [17] have recently demonstrated the utility of poly(vinylidene difluoride) membranes for efficient fraction collection from a CE apparatus without significant sample loss or dilution while maintaining good spatial resolution. The authors where able to separate and collect a protein mixture of myoglobin and β-lactoglobulin. The amount of myoglobin collected on the membrane was 28 pmol, or about 500 ng. Although this is two orders of magnitude higher than the amount of M + 1F detected in our system, presumably the fraction collection system would function satisfactorily for smaller fractions. Furthermore, consecutive CE membrane fraction collections were demonstrated of the separated protein mixture so that successive M + 1F fractions could be harvested from the conjugation reaction mixture. The M + 1F peak is present throughout the first 2 h of the conjugation, therefore at least six successive harvests could be made yielding a total of about 10 ng of product. This method for producing a single label antibody will be investigated by this group in the future.

Acknowledgements

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