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Kinetics and apparent activation energy of the reaction of the fluorogenic reagent 5-furoylquinoline-3-carboxaldehyde with ovalbumin

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Abstract

Incomplete labeling of proteins by a derivatizing reagent usually results in the formation of a large number of products, which can produce unacceptable band broadening during electrophoretic analysis. In this paper, we report on the reaction of the fluorogenic reagent 5-furoylquinoline-3-carboxaldehyde (FQ) with the lysine residues of ovalbumin. Mass spectrometry was first used to determine the distribution in the number of labels attached to the protein. At room temperature, 3.6±1.9 labels were attached after 30 min. The reaction rate and number of labels increased at elevated temperatures. At 65 °C, 6±2.5 labels were attached after 5 min. The apparent activation energy for this reaction is estimated as 48±17 kJ/mol. Based on the mass spectrometry study, the labeling reaction was assumed to consist of two steps. In the first, the protein unfolds to make lysine residues accessible. In the second, the reagents react with the ε-amine of the lysine residues. To test this hypothesis, submicellar capillary electrophoresis and laser-induced fluorescence were used to characterize the reaction mixture. The apparent activation energy was measured for the labeling reaction; the apparent activation energy was 57±12 kJ/mol for reaction performed in the separation buffer. Denaturing agents were added to the reaction mixture. The addition of 2 *M* thiourea with 6 *M* urea to the reaction resulted in a modest decrease in the apparent activation energy to 42±2 kJ/mol. The addition of 2.5 *M* or higher concentration of ethanol decreased the apparent activation energy to 32±2 kJ/mol. We conclude that the apparent activation energy for protein labeling is dominated by denaturation of the protein, and that the addition of suitable denaturing reagents can eliminate this contribution to the reaction chemistry.

Keywords: Kinetics; Apparent activation energy; 5-Furoylquinoline-3-carboxaldehyde; Ovalbumin

1. Introduction

The analysis of the minute amounts of proteins present in a single eukaryotic cell presents a formidable analytical challenge. We have estimated that a typical mammalian cell contains only 50 pg of protein [1], which is many orders of magnitude less material than can be analyzed by conventional gel electrophoresis or mass spectrometry methods. Capillary electrophoresis coupled with laser-induced fluorescence appears to be the only technology that combines both the sensitivity and separation ef-

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ficiency necessary to fingerprint the proteins in a single cell.

Proteins may be detected based on either native fluorescence or fluorescence of a labeled product. Native fluorescence is usually based on emission from tryptophan residues because of their relatively high molar absorptivity, although phenylalanine and tyrosine also generate native fluorescence [2]. Labeling typically requires reaction with lysine or cysteine residues or non-covalent complexation with a fluorogenic reagent. In evaluating the fluorescence detection approaches, it is valuable to consider the composition of a typical protein.

We inspected the yeast proteome, selecting all open reading frames that code for a protein longer than 24 amino acids [3]. We counted the number of amino acids within each translated gene product, and we counted the number of open reading frames that coded for a protein that lacks one of the amino acids. The results are presented in Table 1. The total number of amino acids coded by the yeast genome is 2 889 586. Of course, posttranslational proteolytic digestion often removes a portion of the N-terminal part of the protein; the final protein will contain fewer amino acids than predicted from the gene product. In particular, the start codon codes for an N-terminal methionine residue that is lost in the truncated protein. Nevertheless, the gene product sequence provides insight into the composition of proteins.

Table 1
Amino acid content of open reading frames in yeast

Amino acid	Percentage in yeast proteome	ORFs with amino acid absent
Ala	5.5	40
Arg	4.5	57
Asn	6.1	27
Asp	5.8	78
Cys	1.3	552
Gln	3.9	97
Glu	6.5	59
Gly	4.5	47
His	2.1	210
Ile	6.6	16
Leu	9.6	2
Lys	7.3	20
Met	2.1	0
Phe	4.5	25
Pro	4.3	36
Ser	9.0	4
Thr	5.9	7
Trp	1.0	740
Tyr	3.3	76
Val	5.6	19

The number of each amino acid was determined for all open reading frames coding more than 24 amino acids in *C. cerevisiae*. Data from ftp://genome-ftp.stanford.edu/pub/yeast/data_download/protein_info/.

Tryptophan and cysteine are the least abundant amino acids, each representing only 1% of the amino acids in yeast. These amino acids are absent from roughly 10% of all open reading frames. The translated proteins would not be detected based on tryptophan-based native fluorescence or cysteine-based labeling chemistry.

In contrast, lysine is a quite common amino acid, representing over 7% of all amino acids in the yeast proteome. The median number of lysine residues in a gene product is 26 and the largest number of lysine residues is 369. The translation products of 20 open reading frames lack a lysine residue; only 0.03% of the yeast proteome is inaccessible to lysine labeling chemistry. Most of the open reading frames that do not code for a lysine residue are classified as hypothetical open reading frames. The remainder code for small ribosomal proteins.

Unfortunately, most derivatizing reactions do not go to completion, instead producing a complex mixture of products [4,5]. If there are n possible labeling sites on the protein, then there are $2^n - 1$ possible labeled products. The median yeast protein has 26 lysine residues, which can produce 67 108 863 different possible reaction products. Each product will have a different mobility, and the ensemble can produce a broad peak during electrophoretic analysis [4]. It has been reported that complete labeling of lysine residues is possible [6]. That procedure should generate efficient electrophoresis peaks. However, the procedure is complex and requires a significant amount of sample handling, which is undesirable when handling the proteins in a single cell, and is unlikely to be successful when applied to a protein that contains hundreds of lysine residues.

We have reported that band broadening due to multiple labeling is eliminated by use of the fluorogenic reagent 5-furoylquinoline-3-carboxaldehyde (FQ) to label lysine residues and with a sub-micellar

sodium dodecyl sulfate buffer for electrophoretic analysis of the labeled product [7,8]. The FQ reagent, introduced by Novotny, converts the cationic primary amine to a neutral product [9]. The surfactant appears to ion-pair with the unlabeled residues, which neutralizes the charge so that the protein has the same electrophoretic mobility, irrespective of the number of labels that have been incorporated into the molecule. Through use of an appropriate buffer, separations with >400 000 plates have been obtained for the capillary electrophoresis analysis of FQ-labeled proteins [1,8].

In this paper, we consider the reaction of FQ with ovalbumin. The protein is composed of 386 residues of which 20 are lysine. The protein's N-terminus is acetylated so that the lysine residues are the only primary amines in the molecule [10]. The protein is also phosphorylated at serine residues 69 and 345 and has an N-linked carbohydrate at asparagine residue 293 [11]. Other isoforms have been noted [12,13]. The molecule is highly heterogeneous and provides an excellent model for the complex proteome found in a single eukaryotic cell.

2. Experimental

2.1. Reagents

All buffers were made with Milli-Q deionized water and were filtered using a 0.2-μm filter. Derivatizing reagents, 5-furoylquinoline-3-carboxaldehyde and potassium cyanide, were from Molecular Probes (Eugene, OR, USA). All other reagents were from Sigma–Aldrich (St. Louis, MO, USA) and were used as received.

FQ solutions were dried before storage. A stock solution of 100 m*M* FQ was prepared in methanol, 10 μL aliquots were placed into 500 μL microcentrifuge tubes and the solvent was removed under vacuum using a Speed Vac. The dried FQ was stored at −20 °C. The dried FQ was dissolved in running buffer on the day of the experiment.

2.2. MALDI-TOF analysis of labeled proteins

MALDI-TOF analysis of FQ-labeled ovalbumin was performed with a Hewlett-Packard linear TOF mass spectrometer, Model G2025A. The spectra were the sum of ~50 laser shots.

Ovalbumin samples were labeled by mixing 100 nmol dry FQ, 9 μL of a 25 μ*M* ovalbumin solution prepared in 0.5 m*M* tetraborate buffer, and 1 μL of a 20 m*M* KCN solution in a disposable microcentrifuge tube. A 1 μL aliquot of the reaction mixture was periodically diluted 1:20 in the MALDI matrix solution (13 mg/mL 3,5-dimethoxy-4-hydroxy-cinnamic acid in acetonitrile–H_2O, 45:55) and then 0.5 μL was deposited onto the sample probe. Prior to the application of the sample, 0.5 μL of saturated 3,5-dimethoxy-4-hydroxy-cinnamic acid in acetone was applied to the MALDI probe to provide a sublayer. Each MALDI spot had 0.55 pmol of labeled protein. For the high-temperature reaction, the microcentrifuge tube was maintained at 65 °C in an incubator.

2.3. Fluorescence study of reaction

The capillary electrophoresis instrument was built in-house and used post-column laser-induced fluorescence detection as previously described [14,15]. A 12-mW argon-ion laser beam at 488 nm was used for excitation. Emission was measured at 630 nm through a 30-nm band-pass spectral filter.

A 10^{-7} *M* ovalbumin solution was prepared in a buffer made from 5 m*M* CN^-, 2.0 m*M* tetraborate, and 4 m*M* SDS, pH 9.3. The mixture was injected at 1 kV/cm for 5 s. The capillary tip was dipped twice into a vial containing fresh running buffer to minimize contamination. The FQ solution (5 m*M* FQ in 2.5 m*M* tetraborate and 5 m*M* SDS, pH 9.3) was electrokinetically injected at 1 kV/cm for 5 s. The capillary tip was immersed in a vial of running buffer that had been preheated in a dry bath incubator. After the reaction, the capillary was immersed in running buffer at room temperature. The capillary electrophoresis separation was carried out at 400 V/cm. A 50 μm I.D.×150 μm O.D. capillary with a length of either 35 or 40 cm was used. The running buffer was 2.5 m*M* borax and 5 m*M* SDS at pH 9.3.

2.4. Denaturant study

A $5 \cdot 10^{-5}$ *M* ovalbumin solution was prepared in a 2.5 m*M* borax buffer (pH 9), aliquoted into micro-

centrifuge tubes, and stored at −80 °C. The protein was derivatized by adding 5 μL of 5 m*M* KCN and 10 μL of the specific organic solvent to the dried FQ, followed by 5 μL of the ovalbumin solution, which had thawed on ice. The protein mixtures were allowed to react at room temperature or heated to 65 or 85 °C in an incubator. After reaction, 475 μL of 2.58 m*M* borax was added and the sample was put on ice to quench the reaction. Shortly before performing a CE experiment with the sample, 5 μL of $1 \cdot 10^{-5}$ *M* fluorescein was added, the sample briefly vortexed and centrifuged.

In one case, 2 *M* thiourea plus 6 *M* urea was added to the reaction buffer. In another case, 2.5 *M* ethanol was added. In the third case, the reaction was performed in a 70% ethanol buffer.

2.5. Data analysis

Data were analyzed using Matlab version 6.5 running on a Macintosh computer. Regression analyses were performed using the Curve Fit toolbox.

3. Results and discussion

3.1. MALDI-TOF study of the reaction kinetics of FQ with ovalbumin

Native ovalbumin generated a roughly Gaussian-shaped mass spectrum, centered at 44 200 Da (Fig. 1). The nominal molecular mass of the amino acid content of the protein is 42 881 Da; the N-terminal acetylation and the carbohydrate and phosphate content of the protein contributed about 1300 Da to the observed molecular mass. The 556 Da width of the mass spectrum of ovalbumin is due to the presence of different glycoforms [11].

To study the reaction of FQ with ovalbumin, aliquots of the reaction mixture were periodically taken, quenched by dilution, and then analyzed by MALDI-TOF mass spectrometry. The mean and width of the mass distribution increased with reaction time. The increase in mass resulted from the attachment of an FQ molecule to the ε-amine of lysine residues. The width of the mass distribution also increased with reaction time due to the distribution in the number of labels that had been attached.

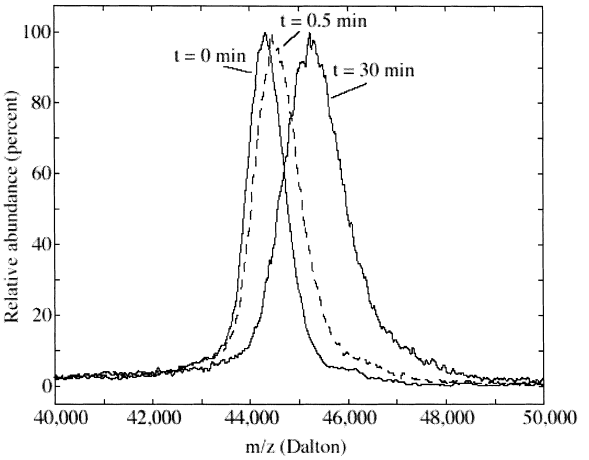

Fig. 1. Mass spectrum of ovalbumin that had been labeled for 0, 0.5, and 30 min at room temperature.

The attachment of a single label increases the mass of the protein by 243 Da, corresponding to the addition of the FQ reagent plus cyanide and the loss of two OH groups during the reaction. If all glycoforms react at the same rate with FQ, then we can model the mass distribution of Fig. 1 as being due to the unlabeled protein plus various amounts of protein with one or more labels. We performed a nonlinear regression analysis where the observed mass distribution was fit with the function

$$intensity(m/z) = \sum_{n=0}^{20} |a(n)| \cdot native(m/z + n \cdot 243) \quad (1)$$

where $intensity(m/z)$ is the observed mass spectrum following labeling. n is the number of labels added to the protein. The addition of one FQ molecule increases the mass of the protein by 243 Da, and $native(m/z + n \cdot 243)$ is the mass spectrum of the reaction product of ovalbumin with n molecules of FQ. $a(n)$ is the fraction of ovalbumin molecules with n labels; its absolute value is used in Eq. (1) to ensure that only positive contributions are considered.

Fig. 2 presents the mass distribution for ovalbumin after a 6-min reaction. We performed a nonlinear fit

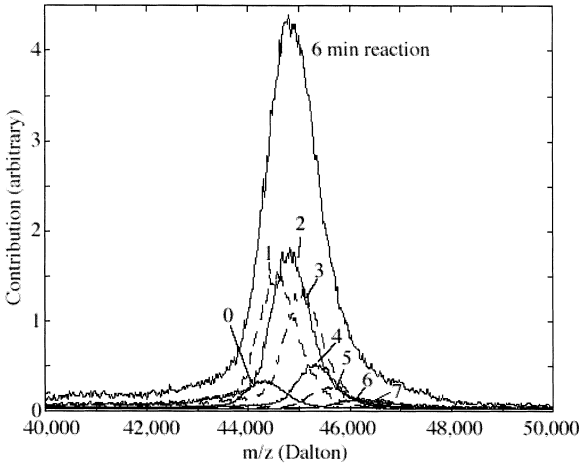

Fig. 2. Deconvolution of the mass spectrum for a 6-min reaction at room temperature. Curves 0–7 are the mass spectra corresponding to the addition of zero to seven labels to ovalbumin.

of Eq. (1) to that spectrum, and the resulting contributions to the spectrum are also presented in the figure. Fig. 3 presents the relative amount of protein with zero to seven labels for the 6-min reaction data. At this reaction time, almost all protein molecules contain at least one label. The most common number of labels was three, but some protein molecules had seven or more labels attached. To estimate the number of labeled sites, we modeled the data with a binomial distribution, where there were 20 possible labeling sites. The smooth curve in Fig. 3 is a binomial distribution with a mean of 2.3 labels and standard deviation of 1.5 labels.

This procedure was repeated for reactions lasting up to 30 min at room temperature and 5 min at 65 °C, at which time the extent of reaction reached a plateau. The average number of labels appeared to follow first-order reaction kinetics (Fig. 4A), with a rate constant of $0.0032 \pm 0.0009\ s^{-1}$ at room temperature. The reaction was an order of magnitude faster at 65 °C (Fig. 4B), where the rate constant was $0.051 \pm 0.005\ s^{-1}$.

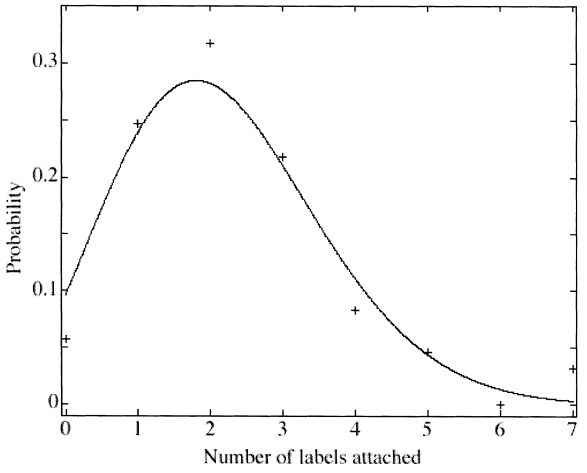

Fig. 3. Relative contributions to the 6-min reaction product from ovalbumin that has had zero to seven labels attached. The smooth curve is the least-squares fit of a binomial distribution to the data. The gamma function was used to interpolate for non-integer labels.

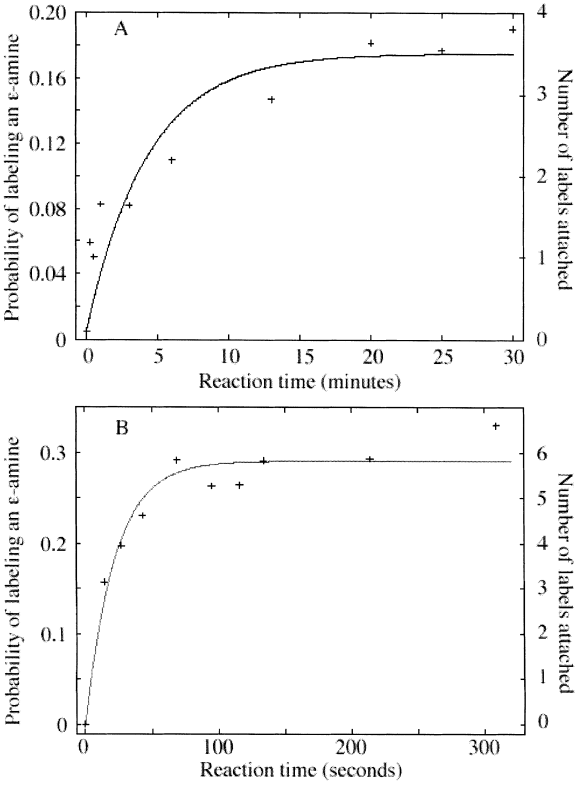

Fig. 4. Probability of labeling an ε-amine versus reaction time. The smooth curve is the least-squares fit of first-order kinetics to the data. The number of labels attached at each time point is presented on the right-hand axis. (A) Room temperature reaction; (B) 65 °C reaction.

At both temperatures, only a few of the 20 possible sites were labeled during the reaction. An asymptotic value of 3.6 labels was attached at room temperature. At 65 °C, the asymptotic value increased to six labels.

Because the reaction did not go to completion, some labeling sites must be inaccessible to the labeling reagent with a correspondingly slow reaction rate. The X-ray crystallographic structure of the partially deglycosylated ovalbumin is available [16,17]. Rasmol was used to measure the distance from each lysine residue to the nearest amino acid (Table 2). Most lysine residues are located in relatively congested portions of the protein structure. However, four residues (residues 39, 69, 78, and 236 in PDB nomenclature) are located 0.49 nm or greater from their nearest neighbor. Presumably, these accessible residues react efficiently at low temperature. As the temperature increases, the protein will partially unfold, which allows more lysine residues to react.

As a result, each lysine residue will have its own reaction rate and activation energy. The reaction is dominated by the behavior of the most labile residues over the initial reaction period. The increase in reaction rate with temperature was consistent with an apparent activation energy of 53±6 kJ/mol for those residues.

Table 2
Distance of each lysine ε-amino group to the nearest residue in ovalbumin

Lysine residue	Distance (nm)	Closest residue	Lysine residue	Distance (nm)	Closest residue
83	0.25	Arg81	191	0.40	Ala343
196[a]	0.26	Glu346	238	0.40	Glu363
199	0.27	Val347	105	0.41	Asn101
285[b]	0.28	Val360	292[b]	0.41	Glu339
283[b]	0.29	Asp356	328	0.43	Phe312
375	0.29	Asn380	271	0.44	Ile266
42	0.30	Glu264	39	0.49	Phe35
216[a]	0.32	Ser390	236	0.49	Ala233
296[c]	0.35	Val72	69	0.53	Gly320
135[b]	0.36	Gln132	78	0.63	Val79

The amino-acid numbering scheme is that used in the Protein Database entry 1OVA.

[a] These residues also have a strong interaction with Glu or Asp.

[b] Most neighboring atoms are part of the protein core.

[c] This lysine is two amino acids away from the carbohydrate attachment site.

3.2. Fluorescence study of the reaction of FQ with ovalbumin

Laser-induced fluorescence provides exquisite detection sensitivity for capillary electrophoresis, extending to the single molecule level in favorable cases [18], and has been used to study protein expression in single cells [1]. However, there is a potential artifact associated with fluorescence labeling of proteins. If the labels are located sufficiently close together, they can interact and quench each other's fluorescence, which eliminates the benefits of efficient protein labeling [19]. We were concerned that the attachment of more than one FQ label on ovalbumin would result in quenching of the fluorescence signal.

To study the effects of multiple labeling on fluorescence detection, ovalbumin was allowed to react with FQ at 21, 65, and 85 °C. Aliquots were periodically withdrawn and the reaction was quenched. The product was then analyzed by capillary electrophoresis with laser-induced fluorescence and the first-order reaction rate was estimated from the data (Table 3). An apparent activation energy of 57±12 kJ/mol was estimated from these data.

Within experimental error, the apparent activation energy determined from fluorescence is identical to that extracted from the mass spectrometry data. This agreement between the fluorescence and mass-spectrometry data demonstrates that the fluorescence signal is not significantly quenched upon addition of several labels to the protein. This result is quite important, because the addition of more labels results

Table 3
Reaction rate ×1000 (s^{-1}) from first-order kinetic fit for reaction of ovalbumin with FQ

Solvent	21 °C	65 °C	85 °C
Borax buffer	0.7±0.1	8±1	53±5
2.5 *M* EtOH	1.3±0.4	6±1	14±4
70% EtOH	0.9±0.2	7±2	9±3
Urea/thiourea	0.4±0.1	4±1	9±3

in a proportional increase in sensitivity and improvement in detection limit.

3.3. Effect of denaturants

The increase in reaction rate with temperature may be due to thermal denaturation of the protein's secondary structure. There are a number of reagents available to denature the protein, and we investigated several.

Urea and thiourea are chaotropes, which disrupt intramolecular hydrogen bonds and lead to protein unfolding [20]. Indeed, addition of a mixture of urea and thiourea did not significantly change the apparent activation energy for the reaction of FQ with ovalbumin, $E_a = 45 \pm 2$ kJ/mol, although the reaction rate decreased at all temperatures.

We also investigated the use of ethanol as a denaturing reagent in the absence of SDS. Ethanol is commonly used as a fixative to preserve tissues before cytometric analysis, and ethanol has been found to be a powerful denaturant of enzyme activity [21]. We investigated both 2.5 *M* and 70% ethanol solutions. In both cases, the apparent activation energy for the reaction of FQ with ovalbumin dropped to 32 ± 5 kJ/mol. The observation of an identical apparent activation energy for 2.5 *M* and 70% ethanol solutions is consistent with the observation that there is a threshold concentration of the solvent required to denature enzymes [21]. This apparent activation energy is similar to the activation energy for the reaction of FQ with low-molecular-mass biogenic amines [22].

We did not investigate the labeling reaction in the presence of proteins that had been treated with reducing agents to disrupt disulfide bonds. This reaction would be challenging to perform on single cells and is beyond the scope of this paper.

3.4. Separation efficiency

Multiple labeling can also degrade separation efficiency in capillary electrophoresis [4,5]. As noted above, the use of FQ with a submicellar separation buffer results in efficient separations. We observed that the number of theoretical plates for the labeled protein increased dramatically with reaction temperature (Fig. 5). As the reaction proceeds for longer time or at higher temperature, more labels are introduced into the molecule, generating a more heterogeneous mixture of labeled product and yielding a complex and broad electropherogram in the absence of anionic surfactant. Krylov has shown that these components can be resolved by isoelectric focusing [23]. However, this distribution in electrophoretic mobility collapsed to a sharp peak upon addition of sub-micellar concentrations of anionic surfactant to the running buffer.

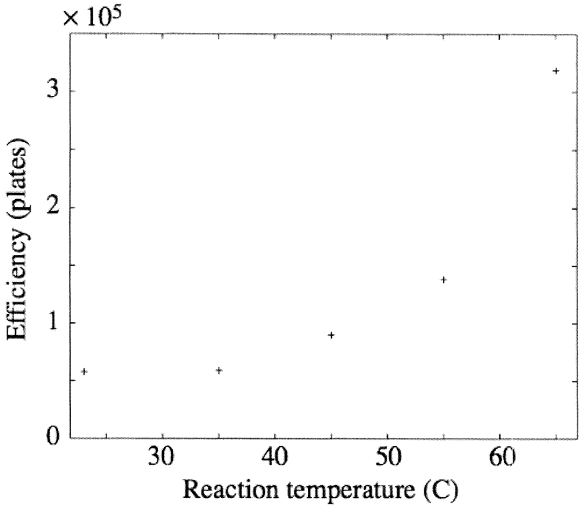

Fig. 5. Number of theoretical plates versus reaction temperature for a 30-s reaction between FQ and ovalbumin.

It would be unfortunate if this collapse in the multiple-labeling envelope was accompanied by co-migration of all labeled proteins. Fortunately, the buffer provides a reasonable separation window. We have used this buffer to analyze complex protein mixtures from human adenocarcinoma cells, and resolved over 20 components in 12 min [1].

4. Conclusion

The apparent activation energy for the reaction of FQ with ovalbumin has two components. One component is associated with unfolding of the protein to make lysine residues accessible to the labeling

reagent. The second component is associated with the reaction of the reagent with the ε-amine of lysine residues. Ethanol serves to both fix tissues, preventing degradation of proteins during storage, and to decrease the apparent activation energy for the labeling reaction. Ethanol also precipitates many proteins at high concentration, and the value of ethanol treatment in single cell analysis is not clear.

Acknowledgements

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