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Accelerator mass spectrometry for assaying irreversible covalent modification of an enzyme by acetoacetic ester

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Abstract

Protein modification (sometimes known as crosslinking) often requires two or more steps to affix a small molecule irreversibly. Two-step reductive alkylation of the enzyme rabbit muscle aldolase with ethyl 3-^{14}C-acetoacetate and sodium cyanoborohydride attaches less radioactivity than with cyanoborohydride omitted. The ^{14}C level incorporated into aldolase corresponds to only about 15–30 modified protein molecules per million. Accelerator mass spectrometry (AMS) provides the only technique currently available for investigating the shorter chains from CNBr-cleavage of modified aldolase. Examination of individual fragments reveals that reductive alkylation of the active site lysine in the presence of cyanoborohydride ($+BH_3CN$) is negligible when compared with the extent of covalent modification in the absence of cyanoborohydride ($-BH_3CN$). Labeling by ethyl acetoacetate cannot result from simple acetoacetylation, because dialysis with hydroxylamine does not wash it out. The amount of ^{14}C incorporated from ethyl 3-^{14}C-acetoacetate without cyanoborohydride is roughly proportional to the number of tyrosine residues in each CNBr-fragment, and we surmise that ethyl acetoacetate attaches irreversibly via a reaction specific to that amino acid. Cyanoborohydride inhibits this reaction, but appears to diminish the susceptibility of the active site tyrosine (which is close to a lysine in the tertiary structure of aldolase) less than other tyrosine residues. (Int J Mass Spectrom 179/180 (1998) 185–193) © 1998 Elsevier Science B.V.

Keywords: Aldolase; Crosslinking; Isotope ratio; Protein; Radiocarbon

1. Introduction

Many proteins operate in living systems by binding smaller molecules (ligands) reversibly. The detailed manner in which proteins respond to ligands and exert their biological effects can be studied by examining small molecules that bind irreversibly. Measuring this interaction often requires isotopic labeling, so that binding can be quantitated through detection of the isotopic tag. Here we report the use of accelerator mass spectrometry (AMS) to quantify the attachment of ^{14}C-containing molecules to an enzyme. We examine irreversible modification of rabbit muscle aldolase with radiolabeled ethyl acetoacetate and look at the amount of radiolabel in the polypeptides derived from subsequent degradation of the labeled protein. The

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Dedicated to Professor Fulvio Cacace in honor of his contributions to mass spectrometry and radiochemistry.

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extent of modification in the fragments correlates with the abundance of a single amino acid and points toward a chemical mechanism for covalent attachment.

Sensitivity and specificity of tracing isotopically tagged compounds depend on both the natural abundance of the isotope and the detection method. Because unstable isotopes occur at low natural abundances, radiotracer experiments have a much greater ultimate sensitivity than experiments using stable isotopes. For example, the terrestrial $^{13}C/^{12}C$ ratio is on the order of 1:90, while the natural abundance of ^{14}C in the biosphere is approximately 1.2 parts per trillion (a level designated as Modern) [1], a level found in living cells that are in equilibrium with the atmosphere. A ^{14}C-labeling experiment can easily have 10^{-10} the background of a ^{13}C-labeling experiment. Moreover, when an organism dies it ceases to equilibrate with atmospheric CO_2, and its ^{14}C decays away. Many commercially available organic compounds are petroleum-derived and routinely contain ^{14}C levels $<0.1\%$ Modern. These can be used as label-free carriers.

Detection methods affect the sensitivity of isotopic tracing. Typical scintillation counters for biochemical quantitation of ^{14}C, with backgrounds on the order of 10 disintegrations per minute (dpm), limit the sensitivity for a ^{14}C label to about 10^{11} atoms ($\sim$150 fmol). The isotopic label stands out well above natural ^{14}C levels but is detected by an inherently inefficient method. No matter what decay counter is used, only eight ^{14}C nuclei will decay per hour for every fmol of ^{14}C present in the sample.

Mass spectrometry offers an efficient method for measuring isotopic label. In analyzing for ^{13}C, the label must be detected by a mass spectrometer that accurately discriminates the isotopic enhancement above its relatively large natural abundance. A fmol of tracer ^{13}C is distinguishable only in samples containing 10 pg or less total carbon. Such small samples can be efficiently ionized for mass analysis, but the variability in natural abundance restricts the sensitivity of stable isotope tracer methods and requires larger sample sizes and ligands labeled with more than one tagged atom. Goodman and Brenna have demonstrated a mass spectrometric detection limit for ^{13}C tracer that approaches 0.25 fmol if multiply ^{13}C-tagged molecules are used [2].

AMS can accurately measure $<10^6$ atoms (.001 fmol) of ^{14}C in a sample containing <1 mg total carbon and can analyze hundreds of samples per day [3]. Sensitivity and small sample size represent major advantages of AMS, but the sample must first be converted to elemental carbon [1]. Because AMS relies upon a highly accurate determination of $^{14}C/^{13}C$ ratios, the technique exhibits an exquisite sensitivity to cross contamination (as well as to adventitious impurities). The technique demands considerable care in purifying and preparing samples of biological origin. Here we describe the application of AMS to measure low levels of covalent modification of a protein by a reagent with high ^{14}C content.

Site-specific covalent attachment of radioligands to proteins allows isolation, purification, and quantitation of the protein with minimal contamination by unbound ligand. One widely studied approach uses reductive alkylation of lysine residues. In the first step the ε-amino groups form Schiff bases with aldehydes or ketones, as Fig. 1 portrays. The term Schiff base designates an iminium ion or its conjugate base (an imine). Because of their positive charge, iminium ions are susceptible to reduction by mild hydride donors, such as cyanoborohydride (BH_3CN^-). As depicted in the lower part of Fig. 1, this yields secondary amines. The net reaction constitutes an irreversible modification [4].

The overall sequence of steps in Fig. 1 is known as reductive alkylation, and it tags lysine residues nonselectively. Some years ago we developed a variant of this reaction that specifically modifies the active site lysine of proteins whose principal biological function is to form Schiff base linkages with neutral ligands [5]. In that procedure the carbonyl compound is an acetoacetic ester, and most proteins do not become irreversibly tagged. However, the same reaction, when applied to the olfactory epithelium of experimental animals, selectively impairs their ability to smell aldehydes and ketones [5,6]. Those results

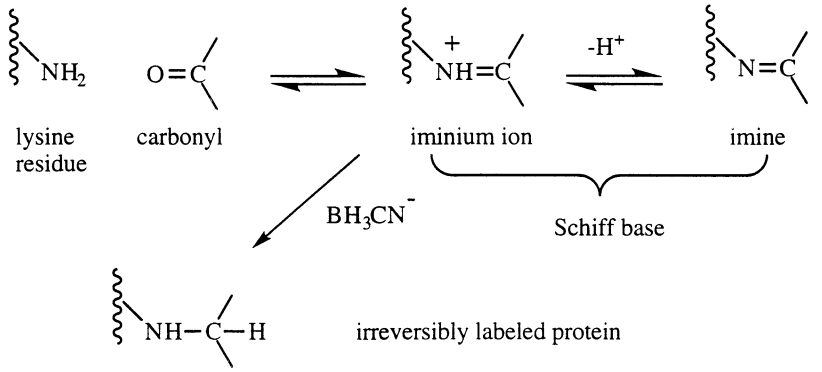


Fig. 1. A widely used scheme for covalently linking a carbonyl-containing ligand irreversibly to a lysine residue within a protein. The reversible formation of the Schiff base renders the iminium ion susceptible to irreversible covalent modification when cyanoborohydride (BH_3CN^-) is added.

imply that Schiff base formation may play a role in olfactory detection of carbonyl compounds.

According to Fig. 1, the net extent of reductive alkylation can be quantified by comparing the incorporation of nondialyzable radiolabel in the presence ($+BH_3CN$) and absence ($-BH_3CN$) of the cyanoborohydride reducing agent. Previously we reported the unexpected result that a Type I aldolase (the most abundant Schiff base-forming enzyme in muscle tissue) is radiolabeled to a greater extent in the control experiment ($-BH_3CN$) [5]. Cyanoborohydride appeared to inhibit labeling of this particular protein, which raises questions as to the origin of the tagging in the $-BH_3CN$ samples and how much reductive alkylation actually takes place in the $+BH_3CN$ samples. Because the extent of labeling for both conditions ($-BH_3CN$ and $+BH_3CN$) was low in the case of aldolase, it was not possible to probe these issues further or to gauge the selectivity of labeling using decay counting.

This paper reports AMS analysis of the ^{14}C distribution in rabbit muscle aldolase tagged by $CH_3{*}COCH_2COOC_2H_5$ (where the asterisk designates the ^{14}C-labeled carbon). The results exemplify an application of a highly sensitive technology that quantifies radioisotope concentrations using mass spectrometric and nuclear identification techniques.

2. Materials and methods

Ethyl 3-^{14}C-acetoacetate (stated specific activity 48 mCi/mmol) was purchased in pentane solution from New England Nuclear, diluted with methanol, and purified by high-performance liquid chromatography (HPLC) on a Microsorb C18 column using isocratic 20% aqueous methanol as the mobile phase. The major ^{14}C-containing impurity, acetone, eluted 3–4 minutes after injection, while the main peak eluted 7 minutes later. For comparing the effects of adding cyanoborohydride versus omitting it, 200 units of rabbit muscle aldolase (~18 mg, EC 4.1.2.13, Sigma Chemical Co.) were dissolved in 1.4 mL 13% aqueous methanol buffered at pH 7.2 with 0.06 *M* HEPES, along with 0.55 μCi freshly purified ethyl 3-^{14}C-acetoacetate and, in the $+BH_3CN$ experiments, 50 m*M* sodium cyanoborohydride. After incubating for 2 h at 37 °C the protein was purified from the reaction mixture by gel filtration on a Pharmacia PD-10 disposable column, with the eluent monitored by BCA protein assay [7] and liquid scintillation counting.

For CNBr digestion 0.2 mL of eluted protein solution (containing no unbound label detectable by scintillation counting) was diluted with 0.1 mL 0.1 *M* HEPES buffer containing 0.3 g guanidine hydrochloride plus 0.8 mg dithiothreitol and incubated 2 h under

argon at room temperature. Then 2 μL 4-vinylpyridine was added, the reaction mixture allowed to stand overnight under argon at room temperature, diluted to 1 mL, and dialyzed against distilled water and lyophilized. The dried protein was then dissolved in 0.5 mL 70% formic acid, 2–3 mg solid cyanogen bromide added, and the solution allowed to stand overnight at room temperature under argon [8]. Prior to electrophoresis, samples of this CNBr digest were lyophilized and then dissolved in TRIS buffer containing 1% sodium dodecyl sulfate (SDS) and mercaptoethanol. The digest was then separated by electrophoresis on 1.5 mm thick 16% acrylamide/0.48% methylene*bis*acrylamide gels at 50 V for 3–4 h with pH 8.25 running buffer containing 0.1 *M* TRIS, 0.1 *M* tricine, and 0.1% SDS [9].

For the hydroxylamine dialysis studies, a separate set of samples of labeled aldolase was prepared as described above without sodium cyanoborohydride and divided into two portions, one of which was dialyzed for 9 h against 50 m*M* hydroxylamine hydrochloride using mw 10 000 cutoff dialysis tubing in 0.05 *M* TRIS buffered at pH 7.2 and the other dialyzed against pure buffer. Intact protein samples were purified by electrophoresis on 12% acrylamide/0.32% methylene*bis*acrylamide under standard Laemmli conditions. Gels were stained for 6–8 h in 2% aqueous phosphoric acid containing 0.1% Coomassie Brilliant Blue G and 6% ammonium sulfate and then rinsed with distilled water.

Horizontal gel slices were placed into quartz tubes, lyophilized, and sealed under vacuum into larger tubes with 0.2 g cupric oxide. After baking at 900 °C for 2 h, the larger tube was opened and CO_2 cryogenically transferred under vacuum to another tube containing 5–8 mg cobalt powder suspended above 0.01–0.02 g TiH_2 and 0.1–0.15 g zinc dust, which was then sealed and baked at 500 °C for 4 h to graphitize the CO_2 [1]. The graphite thus produced was inserted into aluminum sample holders for analysis at the Center for Accelerator Mass Spectrometry. The technique for measuring carbon isotope ratios by AMS has been described in detail elsewhere [3]. Instrumental background is 10^{-6} fmol ^{14}C [10], but processing biological samples limits routine quantification to 0.001 fmol. Raw acrylamide is a petrochemical, and portions of the gel that are well isolated from any protein show a substrate background of 0.002 fmol ^{14}C.

3. Results

Rabbit muscle aldolase reacts with ethyl 3-^{14}C-acetoacetate to incorporate radiolabel irreversibly. Consistent with our previous report [5], more ^{14}C becomes attached when reducing agent (BH_3CN^-) is omitted than when it is present. One possible explanation is that the radioligand is physically entrained by the protein. Denaturation ought to remove the ^{14}C in that case, once the unbound ligand has been separated. Samples were purified by polyacrylamide gel electrophoresis (PAGE) after having been boiled with 1% sodium dodecyl sulfate (SDS) to denature them thoroughly. This standard purification technique (SDS-PAGE) insured that all retained ^{14}C corresponds to covalently attached ligand.

SDS-PAGE bands from loading 15–20 μg of intact protein onto a 1 cm-side lane (visualized with Coomassie Blue) are 2–3 mm wide. These entire bands, as well as comparably sized pieces of unstained gel just before and after the protein bands, were excised and measured by AMS. In most cases it was possible to cut out the bands neatly enough so that the net ^{14}C in the adjacent areas of gel constituted $\leq$5% of the amount in a protein-containing band, and these levels were subtracted from those of the protein bands. Fig. 2 displays the effect of cyanoborohydride on the recovered ^{14}C incorporation, exhibiting nearly twice the level of attachment in the absence of BH_3CN^- than in its presence.

Labeling in the absence of cyanoborohydride might have resulted from covalent attachment to lysine residues via nucleophilic attack of acetoacetic ester by the ε-amino group. The first step of Scheme 1 (where the asterisked carbon denotes the position of the ^{14}C label) illustrates that possibility. This hypothesis can be tested by dialyzing the modified protein with hydroxylamine (H_2NOH), which is known to promote selective hydrolysis of acetoacetamides

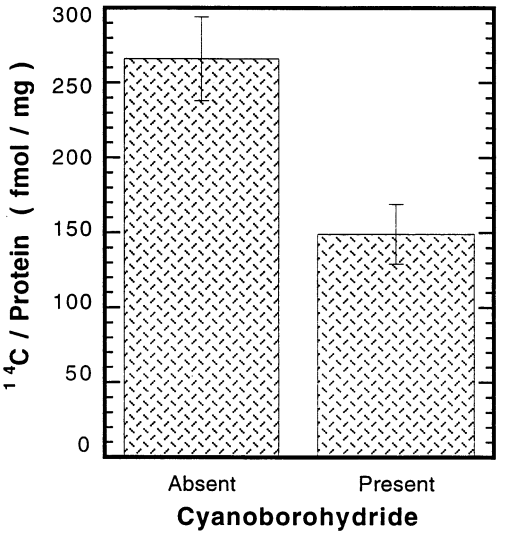


Fig. 2. The effect of added cyanoborohydride on the amount of ^{14}C attached to rabbit muscle aldolase by ethyl 3-^{14}C-acetoacetate, measured after dialysis, denaturation, and SDS-PAGE of the protein. Error bars indicate standard deviations.

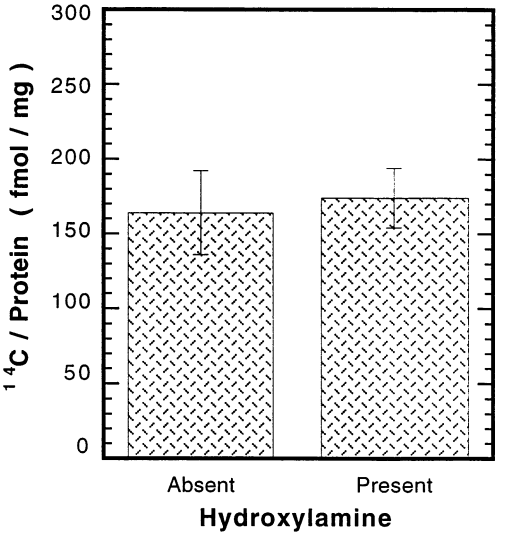
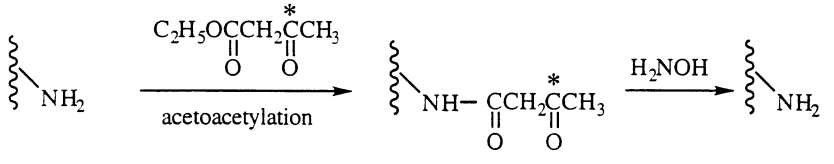


Fig. 3. Lack of effect of hydroxylamine dialysis on the amount of ethyl 3-^{14}C-acetoacetate attached to rabbit muscle aldolase in the absence of cyanoborohydride, measured after dialysis, denaturation, and SDS-PAGE of the protein. Error bars indicate standard deviations.

(which the second step of Scheme 1 represents) [11]. Fig. 3 summarizes a separate labeling experiment, in which dialysis with hydroxylamine had no effect on the ^{14}C content of modified rabbit muscle aldolase. Therefore, we rule out simple acetoacetylation as the labeling mechanism.

To discover whether the labeling exhibits any selectivity, the protein was chemically cleaved into smaller fragments. Tagged aldolase (from the same batch of labeled samples as used for the experiment summarized in Fig. 2) was denatured, the free -SH groups capped with vinylpyridine, and the capped protein subjected to cyanogen bromide (CNBr) in 70% formic acid. At this low pH all basic amino groups are protonated, and the electrophilic CNBr reacts with only the sulfur atoms of methionine residues, leading to selective cleavage at methionine and breaking aldolase into four shorter chains, with molecular weights of 18 kDa, 12 kDa, 8 kDa, and 2 kDa (the 8 kDa fragment contains the active site lysine) [8]. This digest was separated by tricine gel electrophoresis. Visualization required that the gel be loaded with much more protein than needed in the case of the intact enzyme, and the separation distance between bands was not much greater than the widths of the bands themselves. It is thus not surprising that distribution of ^{14}C in the tricine gels was broader than in the SDS-PAGE. Examination of serial slices showed local maxima in ^{14}C content corresponding to the blue bands, but there was substantial crosstalk (which corresponded to a bluish background between



Scheme 1.

Table 1
AMS analyses of irreversible binding of ethyl 3-^{14}C-acetoacetate to peptide fragments from CNBr-cleaved rabbit muscle aldolase as functions of peptide mass and tyrosine content. ^{14}C content is expressed in units corresponding to fmol per mg of intact protein prior to cleavage

	Cyanoborohydride absent				Cyanoborohydride present			
Peptide mol wt	Protein mass (μg) ±10%	^{14}C/peptide (fmol/mg)	s.d. (fmol/mg)	^{14}C/peptide per tyrosine	Protein mass (μg) ±30%	^{14}C/peptide (fmol/mg)	s.d. (fmol/mg)	^{14}C/peptide per tyrosine
18 kDa	32	68	35	23	52	16	8	5
12 kDa	22	125	47	31	34	53	19	13
8 kDa	14	119	25	30	23	15	6	4
2 kDa	4	42	17	42	6	5	2	5

bands). Determining the partition of label among the fragments does not depend significantly upon whether one uses the ^{14}C measured for the bands themselves (the data given in Table 1) or whether the distribution throughout a gel is fit using Gaussian band profiles. We estimate the detection limit of these experiments to be 1.1 fmol ^{14}C per mg of protein (which corresponds to two times the average ^{14}C reading for blank gel), considerably less than our reading for the weakest band. As Table 1 summarizes, every one of the $-BH_3CN$ fragments exhibits more ^{14}C than its respective $+BH_3CN$ fragment. The $+BH_3CN$ band that had the highest level of label corresponded to the 12 kDa fragment, even though the active site lysine resides in the 8 kDa fragment.

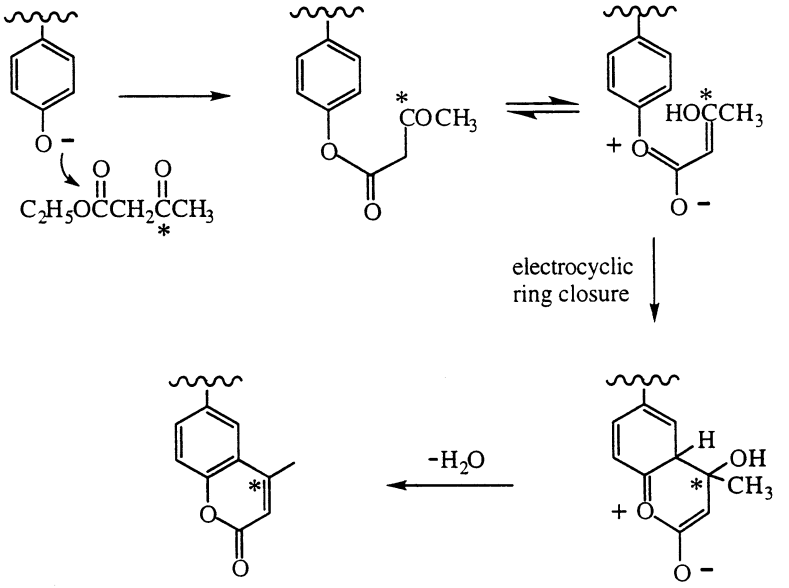
4. Discussion

Accelerator mass spectrometry provides the sensitivity needed to quantitate low levels of ^{14}C incorporation into biological macromolecules [3,12]. AMS permits the analysis of $<$0.1 mg samples of purified protein or protein fragments from gel electrophoresis, where the level of radiolabel is too low to be monitored by decay counting. Rabbit muscle aldolase has a molecular weight of 40 kDa, and these analyses were successfully performed on samples containing $<10^{-14}$ mol of radiolabeled protein.

Treatment of rabbit muscle aldolase with ethyl 3-^{14}C-acetoacetate in the absence of cyanoborohydride ($-BH_3CN$) incorporates radioactivity to the extent of 15–30 μmol per mole of protein after electrophoretic purification. Treatment of enzyme samples with hydroxylamine does not remove ^{14}C that has been incorporated, ruling out simple acetoacetylation as the binding mechanism.

The distribution of ^{14}C among the CNBr-cleavage fragments sets an upper bound for incorporation of ^{14}C via reductive alkylation at the active site lysine. The 8 kDa fragment contains this residue, and the level of incorporation of radiolabel into that polypeptide in the presence of BH_3CN corresponds to $<$1 molecule of ethyl acetoacetate per 10^6 molecules of enzyme. Therefore the level of tagging via reductive alkylation (Fig. 1) is at least an order of magnitude lower than the extent of incorporation by acetoacetic ester in the absence of reducting agent.

The amount of incorporated ^{14}C is not proportional to the molecular weight of the fragments. For example, the ratio of ^{14}C in the 18 kDa fragment to that in the 12 kDa fragment is 0.53 (standard deviation s.d. = 0.12 based on five gels). We have explored whether the extent of labeling in the absence of cyanoborohydride depends on the relative abundance of any one amino acid. The degree of labeling turns out to be proportional to the number of tyrosine residues in each fragment (a plot of the mean level of incorporation gives a linear regression coefficient $r^2 = 0.94$) but does not correlate with the abundance of any other amino acid (in all other cases the regression coefficient is $r^2 < 0.5$). Aldolase contains three tyrosine residues in the 18 kDa fragment, four in the 12 kDa and four in the 8 kDa fragment, and one in the 2 kDa fragment [8]. Schemes 2–4 (where the asterisked

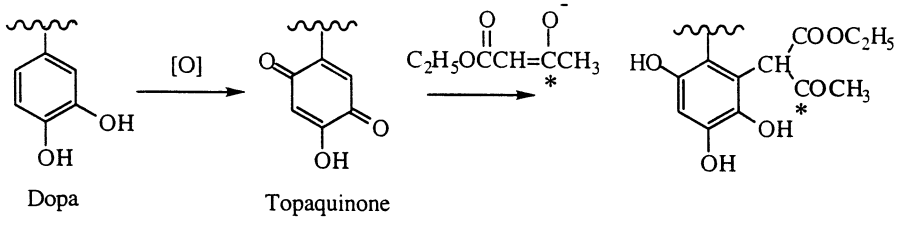


Scheme 2.

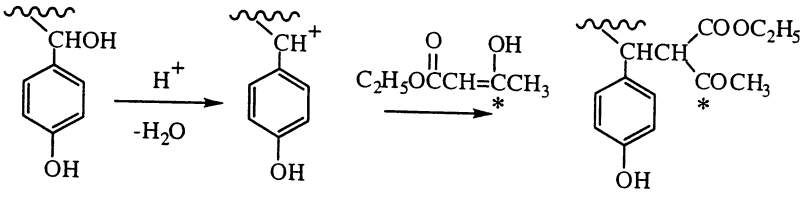
carbon denotes the position of the ^{14}C label) illustrate different hypotheses that could account for selective tagging of the tyrosine sidechain. Scheme 2 depicts a low-yield reaction that might take place on the phenolic group of ordinary tyrosine residues. The ionized hydroxyls first become acetoacetylated. Cyclization then occurs, with net conversion of the phenol to a 4-methylcoumarin. This corresponds to a classical organic reaction, the Pechmann condensation [13], which is ordinarily catalyzed by Lewis acids. Given that the amount of tagging is very low (a level that corresponds to about two out of every 10^6 tyrosine residues), it seems conceivable that an electrocyclic variant of the Pechmann condensation, as drawn in Scheme 2, may occur with a slow rate at physiological pH. The reaction converts the acetoacetylated phenolic sidechain (which can be easily hydrolyzed) to a stable group that resists hydrolysis. In the course of this reaction, the ethyl group is lost from the acetoacetic ester.

One corollary of this mechanism is that suppressing the ionization of tyrosine residues ought to inhibit the acetoacetylation step. Cyanoborohydride is a moderately hydrophobic anion, which could permeate a protein ubiquitously. In that event, proximity of its negative charge should reduce the probability that any given tyrosine is ionized. This would provide an explanation as to why less ^{14}C incorporation takes place in the presence of cyanoborohydride.

The active site of aldolase contains a lysine residue



Scheme 3.



Scheme 4.

in close proximity to a tyrosine [8]. The adjacency of the active site tyrosine to a basic lysine residue suggests that that phenolic sidechain will be more easily ionized than other tyrosine residues. Hence, the 12 kDa fragment (which contains the active site tyrosine) could be more extensively tagged than the others in the presence of cyanoborohydride, even though it contains the same number of tyrosines as does the 8 kDa fragment.

Scheme 3 portrays a different notion of how tyrosines might become modified. This hypothesis supposes that some small fraction of phenol rings have been oxidized to dihydroxyphenylalanine (Dopa) residues (drawn to the left in Scheme 3). Conversion of tyrosine to Dopa is a well-precedented transformation in animal metabolism [14], and it is conceivable that Dopa might be incorporated at a low level into aldolase in place of tyrosine during protein biosynthesis. Dopa becomes easily oxidized nonenzymatically to the corresponding *ortho*-bensoquinone (dopaquinone), which in turn adds a molecule of water and further oxidizes to the quinone derived from trihydroxyphenylalanine (topaquinone) [15]. Topaquinone is susceptible to nucleophilic attack. The enolate of acetoacetic ester (drawn above the second arrow) might plausibly yield adducts such as the one depicted to the right.

Scheme 4 represents an alternative hypothesis, based on the supposition that tyrosines may become oxidized at random at part-per-million levels. In this case the tyrosine residue is supposed to have been hydroxylated at its β-position to form a *para*-hydroxybenzyl alcohol. These are known to be extremely labile in the presence of acid, ionizing to form *para*-hydroxybenzyl cations, which act as vigorous electrophiles [16]. Attack by the enol of acetoacetic ester (drawn above the second arrow in Scheme 3) or its enolate would yield the adduct depicted to the right. For Schemes 3 and 4, the effect of cyanoborohydride could result from its reduction of the aberrant oxidized tyrosine analogues.

Schemes 2–4 represent speculations, but they are testable. If, for instance, acetoacetic ester with a tagged ethyl group incorporates label into aldolase to the same extent as ethyl ^{14}C-acetotacetate, then Scheme 2 must be ruled out. If other reducing agents besides cyanoborohydride fail to suppress labeling, then Schemes 3 and 4 must be ruled out. Experiments to test these hypotheses are underway.

5. Conclusions

The sensitivity of AMS allows determinations of covalent binding on peptide bands separated by conventional analytical gel electrophoresis. Data from individual cleavage fragments permits a search for relationships between the degree of labeling and the occurrence of a given amino acid.

The protocol outlined here has general applicability. A number of small molecules attach to amino acid sidechains with low efficiencies, leading to ubiquitous irreversible modification of a specific amino acid residue. Hitherto, identification of the target site and elucidation of the pertinent chemistry have presented major obstacles, owing to the small amount of isotopic label that incorporates in this fashion. AMS provides the capacity to probe the origin of such “background labeling,” whose mechanistic and physiological relevance can now be explored.

Acknowledgements

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