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Photochemical Nucleophile Mapping: Identification of Tyr311 Within the Catalytic Domain of Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase†

Yasumaru Hatanaka*, Masaki Kaneda and Takenori Tomohiro

Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

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ABSTRACT

Photochemical mapping of nucleophiles in close proximity to the active site Cys149 of rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was demonstrated based on the nucleophilic aromatic photosubstitution reaction using two regioisomers of alkoxy-fluoro-nitro-substituted benzenes. Two photophores were covalently attached to the active site SH group of GAPDH and the protein was subjected to photolysis then to the cyanogen bromide cleavage reaction. The advantage of this method is the capability to chase labeled products by monitoring absorption at 380 nm because of the chromogenic property of photophore. HPLC separation identified a large labeled peptide fragment that was further digested by V8 protease for Edman sequence analysis. From the recent X-ray crystallography of rabbit GAPDH, Tyr311, His176, Ser238 and Lys183 are closely located to catalytic Cys149. Among these nucleophiles, Tyr311 was preferentially labeled with 2-fluoro-4-nitrophenoxy photophore and no label was identified with the isomeric 4-fluoro-2-nitrophenoxy photophore. The result clearly reflects the distance between Cys149 and nucleophiles to distinguish the nearest Tyr311. As photophores show great reactivity even with water under neutral conditions, the distance between nucleophiles and photophores is important for photo-induced nucleophilic aromatic substitution. The method will provide a useful technique to survey nucleophiles within the catalytic domain.

INTRODUCTION

In addition to the active center, enzymes usually require many nucleophilic residues for the specific manipulation of their own substrates. Numerous studies have been reported to elucidate the relationship between activity and nucleophilic residues at the active site. Affinity labeling is one of the most powerful techniques for probing active site nucleophiles based on the selective reactivity of properly designed reactive probes (1); however, the reactivity of affinity probes is unmasked to result in nonspecific labeling of protein surface nucleophiles. On the other hand, photo-affinity labeling uses a chemically inert functional group and irradiation produces very reactive species to nonselectively insert into spatially proximate groups (2,3). Ary azides, phenyl diazirines and benzophenones have been frequently used for photo-affinity labeling. They usually generate a nitrene, carbene or an excited carbonyl, respectively, as a highly reactive intermediate, which allows immediate cross-linking with almost any functional group around the binding site often encountered with analytical difficulties as a result of the multi-site labeling.

Taking the advantage of photo-affinity labeling, the development of a nucleophile-selective photophore should solve the scrambling problem of labeled sites because of the nonselective reactivity of common photophores. To survey and profile nucleophiles in an unknown active center, or to study the function of target nucleophiles by masking or introducing another functional group, we developed a labeling method using photolabile fluoronitrophenoxy group (4). 2-fluoro-4-nitroanisole was reported to react even with water under neutral conditions based on the nucleophilic aromatic photosubstitution reaction mechanism (5). The photochemical nature of 2-fluoro-4-nitroanisole satisfies the requirements of photophores, which are chemically stable before photoactivation, rapidly photolysed and highly reactive to almost all nucleophiles. The nitrophenoxy group also provides the capability to chase labeled products by monitoring absorption at 380 nm because of the chromogenic property of photophores (6,7). In this study, we evaluated fluoronitrophenoxy photophores by probing the active site nucleophiles surrounding the catalytic Cys149 residue of rabbit-muscle GAPDH.

MATERIALS AND METHODS

Materials and instruments. GAPDH was purchased from Roche Molecular Biochemicals, Japan. Kieselgel 60 (70–230 mesh, Merck) was used for column chromatography. All chemicals were of analytical grade and were used without further purification. Melting points were measured on Yamato RD-41 and uncorrected. Amino acid profile was obtained on Hitachi 835, and sequence analyses were performed by Perkin-Elmer Model 477A Protein Sequencer and Model 120A PTH Analyzer. ^{1}H NMR spectra were recorded on JEOL JNM FX-200 spectrometer. UV–vis absorption spectra were obtained on Hitachi M330 and photolysis were performed by UVP Black-Ray MB-100A. Mass spectra (MS) and high-resolution mass spectra (HRMS) were measured on JEOL JMS-300 and JEOL JMS-DX3000, respectively.

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*Corresponding author email: yasu@pha.u-toyama.ac.jp (Yasumaru Hatanaka)

*Synthesis of 2-(fluoronitrophenoxy)ethyl methanethiosulfonate (**1**).* 2-(2-Fluoro-4-nitrophenoxy)ethyl chloride (2.20 g, 10 mmol) and sodium methanethiosulfonate (4.02 g, 30 mmol) were dissolved in 10 mL DMF and the mixture was stirred at 120°C for 1.5 h. To the mixture was added 100 mL benzene, and the precipitate was removed by filtration. After removal of solvent, the product was purified by chromatography on silica gel eluted with benzene. Recrystallization from benzene resulted 2-(2-fluoro-4-nitrophenoxy)ethyl methanethiosulfonate (**1a**) as colorless leaflets, 1.78 g (60%). M.p. 122–123°C. IR (nujor) 1600 cm^{-1}. ^1H NMR ($CDCl_3$) δ 7.7–8.2 (m, 2H), 7.0–7.1 (m, 1H), 4.47 (t, 2H, J = 6 Hz) and 3.44 (s, 3H). MS m/z 295 (M^+). Anal. Calcd. for $C_9H_{10}FNO_5S_2$: C, 36.61; H, 3.41; F, 4.74; N, 6.43; S, 21.71. Found: C, 36.56; H, 3.37; F, 4.74; N, 6.46; S, 21.91. 2-(4-fluoro-2-nitrophenoxy)ethyl methanethiosulfonate (**1b**) was similarly prepared from 2-(4-fluoro-2-nitrophenoxy)ethyl chloride (3.14 g, 20 mmol). Recrystallization from ether yielded colorless leaflets, 3.8 g (65%). M.p. 45–46°C. IR (nujor) 1590 cm^{-1}. ^1H NMR ($CDCl_3$) δ 7.61 (dd, 1H, J = 3, 8 Hz), 7.30 (ddd, 1H, J = 3, 8, 9 Hz), 7.12 (dd, 1H, J = 4, 9 Hz), 4.43 (t, 2H, J = 6 Hz), 3.57 (t, 2H, J = 6 Hz) and 3.40 (s, 3H). MS m/z 295 (M^+). Anal. Calcd. for $C_9H_{10}FNO_5S_2$: C, 36.61; H, 3.41; F, 4.74; N, 6.43; S, 21.71. Found: C, 36.54; H, 3.50; F, 4.79, N, 6.49; S, 21.77.

Preparation of photoreactive GAPDH. Before modification, rabbit-muscle GAPDH was treated with charcoal (Sigma–Aldrich, USA) to remove NAD^+ according to the literature (11). To 0.1 *M* Tris buffer (pH 8.5, 21 mL) containing the resulting apo-enzyme was added 2-(2-fluoro-4-nitrophenoxy)ethyl methanethiosulfonate (**1a**) or 2-(4-fluoro-2-nitrophenoxy)ethyl methanethiosulfonate (**1b**) (660 μ*M* in DMSO), and the mixture was stirred at 4°C for 30 min in the dark to give GAPDH-a and GAPDH-b, respectively. The reaction mixture was subjected to a gel filtration on Sephadex G-25 (1.15 × 30 cm, pyrophosphate pH 8.5, 0.1 m*M* EDTA, 4°C) to remove the excess reagents. The GAPDH fractions were collected by monitoring of absorption at 280 nm and used for the photolabeling experiments.

Photolysis of modified GAPDH and determination of labeling efficiency. A solution of GAPDH-a or GAPDH-b in 10 m*M* pyrophosphate buffer (pH 8.5) containing 0.1 m*M* EDTA was irradiated with a 100 W black-ray lamp for 2 h at 0°C. To determine the efficiency of photo-affinity labeling, 50 m*M* pyrophosphate buffer (pH 8.5, 0.8 mL) and 2-mercaptomethanol (1 μL) were added to a photolabeled GAPDH sample solution (0.2 mL) followed by incubation for 12 h at 20°C. After ultrafiltration through Centricon 10 (Amicon) at 5000 ***g*** for 60 min, UV–vis spectra of the filtrates were measured and the efficiencies were calculated from the absorptions at 400 nm for GAPDH-a and 420 nm for GAPDH-b, respectively.

Treatment of photoproducts with cyanogen bromide. After photolysis of GAPDH-a, the sample solution (40 mL) containing urea (19.6 g, 8 *M*), EDTA (84 mg, 0.2%) and 2-mercaptomethanol (64 μL, 0.9 mmol) was added. The solution was adjusted to pH 8.2 by sodium hydroxide, and incubated at 20°C for 4 h. After incubation with iodoacetic acid (166 mg, 0.8 mmol) at 20°C for 1 h, the mixture was dialyzed in water and then concentrated *in vacuo*. The sample was dissolved in 70% formic acid (27 mL) followed by the addition of cyanogen bromide (95 mg, 0.9 mmol). The mixture was incubated at 20°C for 24 h under argon. To the solution was added water (30 mL) and concentrated to 2 mL *in vacuo*. This procedure was repeated thrice. The solution was centrifuged and the pellet was dissolved in formic acid (1 mL) and combined with the supernatant. The sample solution was loaded on a Chemcosorb 7C18 column (10 × 300 mm, Chemco) which was pre-equilibrated with 26% acetonitrile containing 0.1% trifluoroacetic acid and developed with a linear gradient of acetonitrile consisting of 26–58% over 60 min at flow rate of 2 mL/min. Fractions were monitored by measuring the absorbance at 215 and 380 nm. The main peak fractions were pooled and the solvents were evaporated to 1 mL *in vacuo*. The sample was chromatographed on μ Bondapak C18 (10 × 300 mm, Waters) which had been pre-equilibrated with 0.1% trifluoroacetic acid containing 18% acetonitrile-2-propanol (3 : 7, v/v), followed by a linear gradient of 18–82% acetonitrile-2-propanol (3 : 7, v/v) over 30 min at a flow rate of 1 mL/min. Fractions were monitored by measuring the absorbance at 215 and 380 nm. The main peak fractions were pooled and the solvents were evaporated *in vacuo*. Treatment of GAPDH-b and isolation of the labeled peptides were similarly performed.

V8 protease digestion of photoproducts. The isolated peptides after cyanogen bromide treatment were digested with V8 protease (1 mg/mL, 200 μL, 7.2 nmol) in 100 m*M* sodium phosphate buffer (pH 7.8, 20 mL) at 37°C for 12 h followed by continuous incubation for 36 h after addition of the same amount of V8 protease. The solution was concentrated to 0.5 mL *in vacuo*. The solution was centrifuged and the pellet was dissolved in formic acid (0.3 mL) and combined with the supernatant. The sample was chromatographed on micron Bondapak C18 (10 × 300 mm, Waters) which had been pre-equilibrated with 18% 2-propanol-acetonitrile (7 : 3, v/v) containing 0.1% trifluoroacetic acid, followed by a linear gradient of 18–50% 2-propanol-acetonitrile (7 : 3, v/v) over 30 min at a flow rate of 1 mL/min. Fractions were monitored by measuring the absorbance at 215 and 380 nm. The main peak fractions were pooled and the solvents were evaporated to 1 mL *in vacuo*. The solution was centrifuged and the pellet was dissolved in formic acid (0.5 mL) and combined with the supernatant. The sample solution was loaded on a Chemcosorb 7C18 column (4.6 × 250 mm, Chemco) which was pre-equilibrated with 20% acetonitrile containing 0.1% trifluoroacetic acid and developed with 20% acetonitrile containing 0.1% trifluoroacetic acid at flow rate of 1 mL/min. After removal of the solvent, the residue was dissolved in 70% formic acid for amino acid sequence analysis. Treatment of GAPDH-b and purification of the labeled peptides were similarly performed.

Photoreaction of fluoronitroanisoles and N-acetyllysinamide. 2-Fluoro-4-nitroanisole (**2a**, 171 mg, 1 mmol) and *N*-acetyllysinamide (187 mg, 1 mmol) was dissolved in a mixed solvent (20 mL) of 50% (v/v) acetonitrile and 0.1 *M* aqueous sodium hydrogen carbonate. The solution was irradiated with a 100 W black-ray lamp for 5 h under nitrogen. After removal of the solvent, the product was chromatographed on silica gel (chloroform : methanol = 20 : 1) followed by recrystallization from ethyl acetate to give *N*-acetyl-*N*′-(2-methoxy-5-nitrophenyl)lysinamide (**3a**) as yellow needles (12 mg, 4%). M.p. 184–185°C. IR (nujor) 3320, 3200, 1700, 1680 cm^{-1}. ^1H NMR ($CDCl_3$) δ 7.62 (dd, 1H, J = 3, 9 Hz), 7.34 (d, 1H, J = 3 Hz), 6.74 (d, 1H, J = 9 Hz), 6.07 (br s, 2H), 5.39 (br s, 1H), 4.48 (dt, 2H, J = 7, 7 Hz), 4.39 (br s, 1H), 3.94 (s, 3H), 3.21 (dt, 2H, J = 7, 7 Hz), 2.03 (s, 3H) and 1.4–2.0 (m, 6H). MS m/z 388 (M^+). HRMS m/z calcd. for $C_{15}H_{22}N_4O_5$: 388.1592. Found: 338.1602. 2-Methoxy-5-nitrophenol (21 mg, 12%) was also given in the reaction and **2a** (137 mg, 80%) was recovered. The photoreaction of 4-fluoro-2-nitroanisole (**2b**) for 10 h gave *N*-acetyl-*N*′-(4-methoxy-3-nitrophenyl)lysinamide (**3b**) as orange needles after recrystallization from ethyl acetate (26 mg, 8%). M.p. 132–133°C. IR (nujor) 3350, 3200, 1700, 1675 cm^{-1}. ^1H NMR ($CDCl_3$) δ 7.05 (d, 1H, J = 3 Hz), 6.94 (d, 1H, J = 9 Hz), 6.78 (dd, 1H, J = 3, 9 Hz), 6.09 (br s, 2H), 5.42 (br s, 1H), 4.51 (dt, 2H, J = 7, 7 Hz), 3.88 (s, 3H), 3.72 (br s, 1H), 3.12 (t, 2H, J = 7 Hz), 2.03 (s, 3H) and 1.4–2.0 (m, 6H). MS m/z 388 (M^+). HRMS m/z Calcd. for $C_{15}H_{22}N_4O_5$: 388.1592. Found: 338.1602. 4-Methoxy-3-nitrophenol (31 mg, 18%) was also given in the reaction and **2b** (116 mg, 67%) was recovered.

RESULTS AND DISCUSSION

Preparation of photoreactive GAPDH

GAPDH is a homo-tetramer protein and each subunit has an active site containing an un-oxidized sulfhydryl group of Cys149 used for catalytic reaction. It catalyzes the oxidation of glyceraldehyde-3-phosphate to form 1,3-diphosphoglycerate with the concomitant reduction of β-nicotinamide adenine dinucleotide (NAD^+) (8–10). The chemical labeling of the rabbit-muscle GAPDH active site with thermally reactive probes suggested that the ϵ-amino group of Lys183 could be near to catalytic SH because it was acetylated through the migration of acetyl Cys149 (15) from *S* to *N* atom and this observation was confirmed by a cross-linking reagent (16). The X-ray structure of rabbit-muscle GAPDH was recently reported (11), and the catalytic center was revealed as the first detailed model of mammalian GAPDH. There are several

nucleophiles existing within 6 Å from the active site Cys149 of rabbit-muscle GAPDH, such as His176 and Tyr311, whereas the distance to Lys183 is about 3 times longer than these nucleophiles. For the photochemical nucleophile mapping of rabbit-muscle GAPDH, fluoronitrophenyl derivatives having a SH-reactive methanethiosulfonyl group (**1a** and **1b**, Fig. 1) were designed for attaching photophores to the catalytic Cys149 *via* disulfide linkage (12). Because nucleophilic aromatic photosubstitution takes place at the fluorinated position of the benzene ring, *ortho*- and *para*-derivatives were prepared to compare labeling efficiency and ability depending on the distance from Cys149 to nucleophiles.

As bound NAD^+ inhibits the modification of the Cys149 SH group (13), the cofactor was removed before the incorporation of photoreactive moiety. The reaction of apo-GAPDH and compound **1a** or **1b** efficiently proceeded at 4°C to give photoreactive GAPDH proteins (GAPDH-a and GAPDH-b, respectively). The reaction was monitored by measuring the enzyme activity of GAPDH according to the literature (9). GAPDH activities after 30 min were 0.08 and 0.03% of the original activity by the reaction of compounds **1a** and **1b**, respectively. The electronic absorption spectra of photoreactive GAPDH-a and -b are shown in Fig. 2 (spectra at 0 min). The modification yield was calculated based on absorption because of the fluoronitrophenoxy group by comparison with the values of glutathione derivatives of compounds **1a** and **1b** [λ_{max} 314 nm (ϵ 11500) and 343 nm (ϵ 2630), respectively] as 1.01 and 0.99 molar ratios of fluoronitrophenoxy groups per

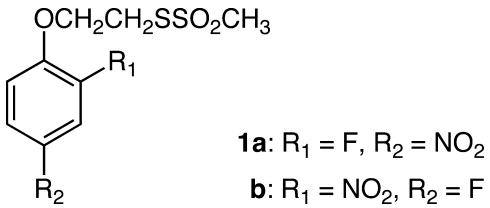

Figure 1. 2-(Fluoronitrophenoxy)ethyl methanethiosulfonate compounds as photoreactive reagents with a disulfide linkage.

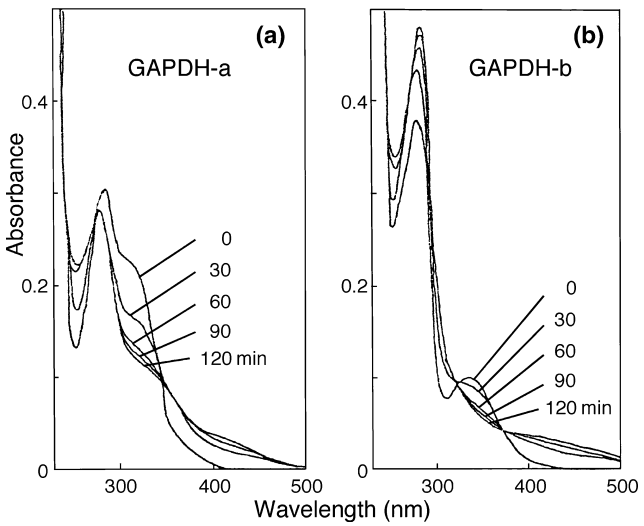

Figure 2. UV–vis spectral changes (240–500 nm) of photoreactive GAPDHs by irradiation are shown. (a) GAPDH-a modified by 2-(2-fluoro-4-nitrophenoxy)ethyl methanethiosulfonate. (b) GAPDH-b modified by 2-(4-fluoro-2-nitrophenoxy)ethyl methanethiosulfonate.

one subunit of GAPDH-a and -b, respectively. The concentration of GAPDH protein itself was determined from the absorption at 278 nm.

Photoreactions of modified GAPDH

Photolysis was performed at 0°C with a 100 W long-wavelength UV lamp until specific absorption as a result of photoreactive moieties, fluoronitrophenoxy groups, disappeared. Figure 2 shows UV–vis spectra of photoreactive GAPDHs and the time course of degradation of photoreactive groups by irradiation. Absorption around 310–320 nm for GAPDH-a and 320–340 nm for GAPDH-b clearly decreased with the irradiation time. These results show that photolysis was completed within 120 min. The electrophilic intermediates produced during photolysis should mainly react with spatially proximate nucleophiles at the active site of GAPDH to form covalent bonds with amino acid residues. However, photoreactive fluoronitrophenoxy groups also reacted with the surrounding water molecules and were converted to nitrophenol derivatives. After treatment with 2-mercaptoethanol to cleave the disulfide bond, nitrophenol derivatives should be separated and isolated by ultra-filtration from the protein. The electronic absorption spectra of the filtrates were measured to determine the amount of 'unlabeled' nitrophenol derivatives and the labeling yields were then calculated based on the ϵ values of 2-hydroxy-4-nitroanisol (**4a** for GAPDH-a) and 4-hydroxy-2-nitroanisol (**4b** for GAPDH-b) to give 9% and 12%, respectively.

Identification of the photolabeled sites

The photolabeled proteins were then subjected to amino acid analysis of the labeled fragments and determination process of the labeling sites.

Firstly, photolabeled GAPDH-a and GAPDH-b were cleaved with cyanogen bromide after reductive carboxymethylation. The resultant fragments were purified by HPLC by monitoring electronic absorption at 215 and 380 nm. As a nitrophenoxyl group shows absorption around those wavelengths, the labeled peptide tethering it can be pursued by spectral analysis. A broad band eluted around 28–32 min was isolated (Fig. 3a) and subjected to amino acid analysis. The chromatograms showed that the isolated fragments contained many acidic residues and a large amount of unlabeled peptides. The fragments were then digested by V8 protease and the proteolytic fragments were separated by HPLC followed by isolation of the fragment eluted at 28 min (indicated by arrow in Fig. 3b) that showed absorption at 380 nm. A similar result was obtained for GAPDH-b. The result of Edman sequence analysis for GAPDH-a fragments is summarized in Table 1. It showed that the photolabeled peptide was equivalent to residues 303–314. The amino acid residue 310 (cycle 8) was analyzed as alanine instead of tryptophan. This region is well conserved from bacteria to mammals and the residue at this position was already identified as tryptophan (11, see SUPPLEMENTAL MATERIALS). As it was reported that tryptophan residue was oxidized by cyanogen bromide (14), a similar reaction should occur in this case. Furthermore, the product of cycle 9 was not detected at the expected retention time of PTH-Tyr derivative on HPLC (see SUPPLEMENTAL MATERIALS). The result suggested that Tyr311 in GAPDH

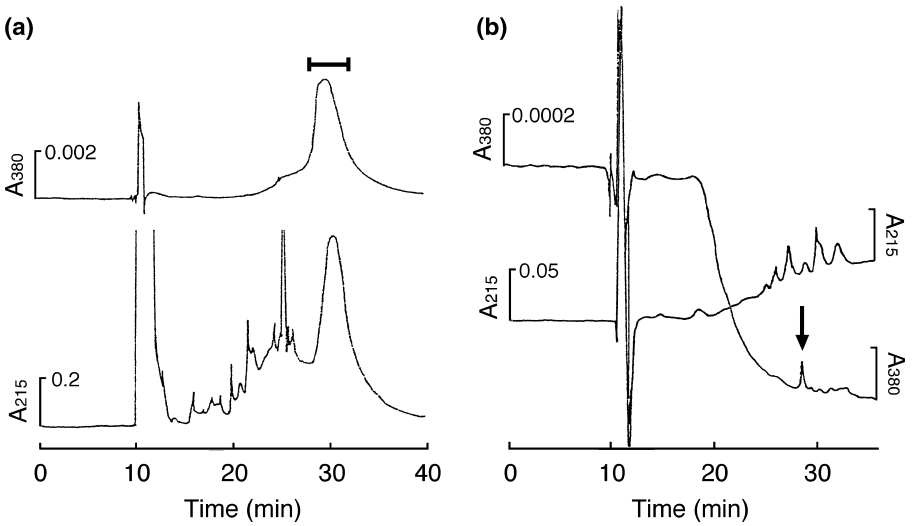

Figure 3. Chromatogram profiles of proteolytic products of photolabeled GAPDH-a. The eluates were chased by monitoring absorptions at 215 and 380 nm. (a) Chromatogram of degradation sample of photolabeled GAPDH-a with cyanogen bromide. The fraction indicated by the bar was collected and subjected to amino acid analysis. (b) Chromatogram of the digestion sample of photo-labeled GAPDH-a with V8 protease. The indicated fraction (arrow) was collected for Edman sequence analysis.

Table 1. Determination of amino acid sequence of the labeled peptides for position 303–314

	PTH amino acids (pmol)	
Cycle	V8 fragment-a	V8 fragment-b
1	His (220)	His (44)
2	Phe (1144)	Phe (752)
3	Val (1249)	Val (489)
4	Lys (560)	Lys (212)
5	Leu (1024)	Leu (462)
6	Ilu (1023)	Ilu (397)
7	Ser (105)	Ser (24)
8	Ala (144)	Ala (159)
9	Xaa (–)[a]	Tyr (71)
10	Asp (189)	Asp (41)
11	Asn (262)	Asn (35)
12	Glu (30)	Glu (20)

[a]Not detected.

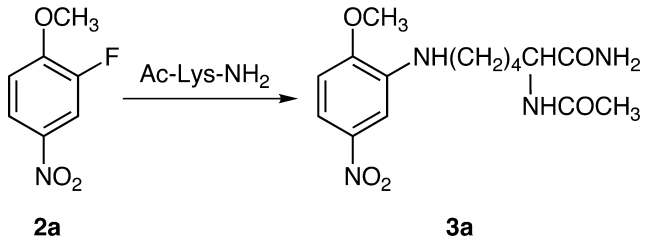

Figure 4. Photoreaction of 2-fluoro-4-nitroanisole with *N*-acetyllysinamide.

was labeled by 2-fluoro-4-nitrophenoxy group upon photolysis. In the case of GAPDH-b, the amount of PTH derivatives was rather small such as Ser309, Asp312, Asn313 and maybe Tyr311 (Table 1). However, the labeled amino acid residues were not clearly detected in this study, although the isolated peptide showed absorption at 380 nm.

As Tyr311 was labeled in this study, the photochemical reactivity of fluoronitroanisole derivatives, 2-fluoro-4-nitroanisole (**2a**) and 4-fluoro-2-nitroanisole (**2b**), was investigated with *N*-acetyltyrosinamide for confirmation (see SUPPLEMENTAL MATERIALS). The photoreactions of fluoronitroanisoles and *N*-acetyltyrosinamide were carried out in 0.1 *M* carbonate buffer (pH 8.0) - acetonitrile (1: 1) under an inert atmosphere. Both reactions slowly proceeded, resulting in the recovery of more than 75% of starting material after 10 h irradiation. The products were mainly corresponding phenol derivatives derived from the reaction with water, and no tyrosine adducts were detected. Although the reactions were examined in dimethylformamide or higher basic solution (pH 12), similar results were obtained. On the other hand, the photoreaction using *N*-acetyllysinamide was also studied (Fig. 4) because it has an amino group that is usually considered as a more reactive nucleophile than phenol group. In addition, it has been suggested that *S* to *N* migration of the acetyl group from Cys149 to Lys183 could proceed via an intermediary group (15,16). Lysine adducts were detected and isolated in 4% and 8% yields for compounds **3a** and **3b**, respectively. These results indicate that nucleophilic substitution of the photophores depends on the basicity of nucleophiles, and the competitive quenching reaction with water is sufficiently slow even under basic conditions. While the labeled peptide was unfortunately not identified for GAPDH-b, only Tyr311 was clearly labeled in the photolysis of GAPDH-a. In terms of the reactivity of the fluoronitrophenoxy group against tyrosine, it was suggested that 2-fluoro-4-nitrophenoxy groups should exist spatially proximate to Tyr311. Actually, Fig. 5 shows that the distance between Cys149 and Tyr311 is 4.9 Å, estimated from the X-ray structure (11), comparable to *ca* 6 Å of the distance between Cys149 and the *ortho*-position (reaction site) of the benzene ring. In the case of the 4-fluoro-2-nitrophenoxy group, substitution at the *para*-position of the benzene ring by tyrosine residue is supposed to be sterically and conformationally difficult because of the rather long distance between the *para*-position and Cys149 (*ca* 8 Å) as well

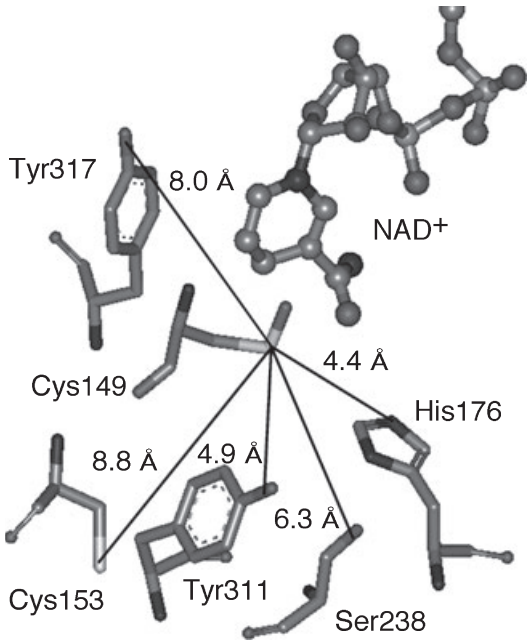

Figure 5. Relationship between the oxidized active site of Cys149 and surrounding amino acid residues as well as the adenine part of the cofactor NAD^+ (PDB ID: 1J0X (11)).

as the rigidity of photophores. These results obtained by photolabeling obviously reflected the three-dimensional alignment of nucleophiles within the active site. Although the amino group of lysine showed much higher reactivity, Lys residue did not react with photophores in this experiment. The distances to Lys183 and Lys306 from Cys149 are 17.8 and 13.5 Å, respectively, which are too far to make a bond. Furthermore, there are other nucleophiles spatially proximate to Cys149, including His176 (4.4 Å), Ser238 (6.3 Å), Try317 (8.0 Å), Cys153 (8.8 Å), as well as two adjacent amino acid residues, Ser148 and Thr150. In a previous report, the 2-fluoro-4-nitorophenoxy derivative of glutathione was successfully identified as a histidine residue within the substrate-binding site of *S*-transferase (17). No labeled products of these residues, including His176, were detected at this time, although His176 exists close to Cys149 as well as Tyr311.

CONCLUSION

We presented an example of nucleophile mapping based on a nucleophilic aromatic photosubstitution reaction and demonstrated that Tyr311 of rabbit-muscle GAPDH was mainly photolabeled by a photoreactive and chromogenic 2-fluoro-4-nitrophenoxy group attached to the active site of Cys149. Substitution took place with Tyr residue although it showed lower reactivity than Lys residue. In addition, labeling of spatially proximate His176 did not occur in this study, since the substitution reaction by His residue was already reported. The result suggested that the distance and configuration of a nucleophile to photophores must be of primary importance for labeling.

In this study, a new method of photolabeling has been developed for the mapping of nucleophiles located in the proximity of fluoronitrophenoxy photophores.

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SUPPLEMENTAL MATERIALS

Figures S1, S2 and Table S1 can be found at www.blackwell-synergy.com

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