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Identification of the Arylazido-β-alanyl-NAD$^+$-Modified Site in Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase by Microsequencing and Fast Atom Bombardment Mass Spectrometry†

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Received February 28, 1986; Revised Manuscript Received May 2, 1986

ABSTRACT: We have identified the site labeled by arylazido-β-alanyl-NAD$^+$ (A3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$) in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by microsequencing and fast atom bombardment mass spectrometry. This NAD$^+$ photoaffinity analogue has been previously demonstrated to modify glyceraldehyde-3-phosphate dehydrogenase in a very specific manner and probably at the active site of the enzyme [Chen, S., Davis, H., Vierra, J. R., & Guillory, R. J. (1984) *Biochem. Biophys. Stud. Proteins Nucleic Acids, Proc. Int. Symp., 3rd*, 407–425]. The label is associated exclusively with a tryptic peptide that has the sequence Ile-Val-Ser-Asn-Ala-Ser-Cys-Thr-Thr-Asn. In comparison to the amino acid sequence of glyceraldehyde-3-phosphate dehydrogenase from other species, this peptide is in a highly conserved region and is part of the active site of the enzyme. The cysteine residue at position seven was predominantly labeled and suggested to be the site modified by arylazido-β-alanyl-NAD$^+$. This cysteine residue corresponds to the Cys-149 in the pig muscle enzyme, which has been shown to be an essential residue for the enzyme activity. The present investigation clearly demonstrates that arylazido-β-alanyl-NAD$^+$ is a useful photoaffinity probe to characterize the active sites of NAD(H)-dependent enzymes.

In recent years, photoaffinity labeling techniques have been widely applied to characterize enzyme active sites. However, the usefulness of these techniques has been seriously limited for several reasons. In many investigations, difficulties were found in the characterization of the modified peptides. It is not uncommon to find that modification changes the chemical and/or physical properties of peptides and complicates the separation and structural analysis. Additionally, the attachment can be labile, and the modifying groups may be lost during the characterization process. Also, the amounts of the modified peptides may simply be too low for full characterization. Therefore, most of the studies have only been carried out as far as demonstrating the selectivity and specificity of reaction between the photoprobes and enzymes. Only a few modified active-site peptides are actually characterized. In most instances, these photoprobe-labeled peptides are identified indirectly by comparison of the amino acid composition of the modified peptides with those of the peptides with known sequences. Even in those cases where the modified peptides have been identified and the modified amino acids have been determined, there is always some uncertainty in the interpretation of the results. The question is constantly raised whether the photoprobe indeed binds to the active site, to regions near the action site, or even to regions not directly related to the activity. Because most photoprobes are synthesized by modifying the structure of the natural ligand or by adding a photoreactive group, it is reasonable to rationalize that these structural changes may have some influence on their binding to the active site. Alternatively, the probe can bind to a specific site other than the active site (e.g., allosteric site). It is also possible that the probe binds to regions resulting from a postphotolyzed interaction, if the photoactivated species has a lifetime sufficient to allow it to diffuse away from the ligand binding site before the insertion takes place. Thus, it is clear that, in order to take full advantage of the photoaffinity labeling techniques in the investigation of the active site of enzymes, it is necessary to have sensitive and direct methods to characterize the modified peptides and to understand the nature of the interaction of photoaffinity probes with the enzymes.

The recent development of gas phase microsequencing techniques allows protein/peptide sequencing at the subnanomole range (Hewick et al., 1981; Hawke et al., 1985). In addition, fast atom bombardment mass spectrometry

† Supported in part by NIH Grant HD 14900.
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0006-2960/86/0425-5391$01.50/0

(FAB/MS)[1] has been useful in providing accurate molecular weight values for intact peptide and sequence-related fragments (Lee, 1986). The present study illustrates the usefulness of combining microsequencing and FAB mass spectral techniques for identifying the peptide modified by arylazido-β-alanyl-NAD^+ (aaNAD^+) in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and determining with certainty the amino acid to which the probe is attached.

Arylazido-β-alanyl-NAD^+ is a photoaffinity analogue of NAD^+. It has been shown to be a potential active-site-directed photolabel for a number of NAD(H)-dependent oxidoreductases (Chen & Guillory, 1977, 1981, 1984a; Chen et al., 1984; Yamaguchi et al., 1985). It has been found to be a substrate or competitive inhibitor of these enzymes in the dark and labels the enzymes in a specific and stoichiometric manner upon photoirradiation. However, the exact nature of aaNAD^+ attachment in these enzymes is not yet known.

Arylazido-β-alanyl-NAD^+ is a good substrate for rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (its K_m and V_{max} values are 288% and 59%, respectively, of those for NAD^+) (Chen et al., 1984). The association of only 1 mol of aaNAD^+ per mole of enzyme resulted in 100% inactivation of enzymatic activity upon photoirradiation, indicating that aaNAD^+, like NAD^+, binds to the enzyme in a negatively cooperative fashion (Chen et al., 1984). Glyceraldehyde-3-phosphate dehydrogenase is a well-characterized NAD(H)-dependent enzyme. The primary structures of the enzyme from several different sources have been determined, and a significant homology among these sequences has been found (Harris & Waters, 1976; Walker et al., 1980). The X-ray structures of the enzymes isolated from lobster muscle (Watson & Banuszak, 1964; Moras et al., 1975; Marthy et al., 1980), from human muscle (Gorjunov et al., 1972; Watson et al., 1972), and from *Bacillus stearothermophilus* (Biesacker, 1977) have been determined and found to be very similar. A detailed structure of the active site of this enzyme has been presented (Moras et al., 1975; Biesacker, 1977).

Because the active site of glyceraldehyde-3-phosphate dehydrogenase is well characterized, it is felt that this enzyme should be a good model system to investigate the binding nature of aaNAD^+ to NAD(H)-dependent enzymes. By relating findings from photolabeling experiments with available information concerning the structure of the active site of the enzyme, one will be able to better understand the nature of the interaction.

Materials and Methods

Materials. β-[3-^{3}H]Alanine (specific activity 86.4 Ci/mmol) was obtained from New England Nuclear Corp. Arylazido-β-alanyl-NAD^+ and arylazido-β-[3-^{3}H]alanyl-NAD^+ were synthesized as described by Chen and Guillory (1977). Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was obtained from Sigma Chemical Co.

Enzymatic Assay. Glyceraldehyde-3-phosphate dehydrogenase activity was determined spectrophotometrically by measuring at 25 °C the reduction of NAD^+ at 340 nm. The assay condition is identical with that reported by Chen et al. (1984).

Preparation and Trypsin Digestion of Arylazido-β-alanyl-NAD^+-Modified Glyceraldehyde-3-phosphate Dehydrogenase. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (2.6 mg in 1 mL of 100 mM ammonium bicarbonate, pH 7.8) was first subjected to the Sephadex G-25 syringe column centrifugation (in the same buffer) by a procedure described by Chen and Guillory (1981) to remove endogenous NAD(H). The enzyme preparation was then photolyzed for 2.25 min in the presence of 0.15 mM [^{3}H]-aaNAD^+ (specific activity 1.85×10^7 cpm/μmol). After addition of 0.13 μmol of nonradioactive aaNAD^+ (in 70 μL of distilled water), it was photolyzed again for 0.75 min. The photolyzed sample was once more applied to the Sephadex G-25 syringe column to remove the unbound analogue derivatives. The enzyme activity and the bound radioactivity were determined with small aliquots of the eluent. It was found that this sample was 94% inhibited with a total bound radioactivity of 2×10^5 cpm.

An additional sample was prepared by performing the [^{3}H]aaNAD^+ labeling in the presence of 1 mM NADH. It was found that, after removing the unbound ligand, this latter sample contained a total bound radioactivity of 3.5×10^4 cpm associated with a 32% inhibition of the activity.

These two samples, together with a control sample (i.e., the enzyme preparation photolyzed in the absence of aaNAD^+), were digested overnight at 37 °C with 100 μg of trypsin.

Separation of Peptides by Reverse-Phase HPLC. The peptides were first separated on a Vydac C-18 analytical column, and the radioactive peptide was further purified on an Altex Ultrasphere ODS analytical column. Chromatographic conditions are similar to those described by Yuan et al. (1982): a 120-min linear gradient from solvent I (0.1% TFA) to solvent II (0.1% TFA/90% acetonitrile).

Analysis of Isolated Peptides. The isolated radioactive peptide was characterized by amino acid analysis, sequence analysis, and FAB/MS analysis. Amino acid compositions were determined on a Beckman 121 MB amino acid analyzer after hydrolysis in 5.7 N HCl at 110 °C for 24 h, and cysteine content was determined as cysteic acid after performic acid oxidation. Automated sequence analysis was performed on a gas phase sequencer built at this institute (Hawke et al., 1985). Positive ion FAB mass spectra of the peptide fraction were taken with a JEOL HX100HF double-focusing mass spectrometer operating at 5-kV accelerating potential and using a 3-kV xenon atom beam. The sample peptide (50–100 pmol) in 2 μL of 5% aqueous acetic acid was added to approximately 1 μL of dithiothreitol/dithioerythritol (5:1) on a 1.5 × 6 mm stainless-steel sample stage. Repetitive scans over the range m/z 380 to m/z 3500 (cycle time = 40 s) were collected, and mass was assigned by using a JEOL DA5000 data system. Mass assignments are accurate to ±0.2 amu.

Results and Discussion

By the procedure described under Materials and Methods, 2.6 mg of [^{3}H]aaNAD^+-labeled glyceraldehyde-3-phosphate dehydrogenase was prepared. Figure 1A shows the chromatogram of the tryptic digests of the labeled enzyme by reverse-phase HPLC on a Vydac C-18 column. Only one radioactive peak associated with the peak T-16 was found. It was also noticed that T-16 was the only peak in the chromatogram at that sensitivity with a higher 214-nm absorbance compared to the chromatogram of the tryptic digest of the control preparation (Figure 1B). The chromatogram of the tryptic digest of the sample that was photolyzed with aaNAD^+ in the presence of NADH (see Materials and Methods) resembled more closely that of the control, and a lower amount of the radioactive probe was incorporated (results not shown). Because the chromatograms from all three samples contained this peak, it was clear that T-16 was not a pure preparation

[1] Abbreviations: aaNAD^+, arylazido-β-alanyl-NAD^+, A3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl]NAD^+; FAB/MS, fast atom bombardment mass spectrometry; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin.

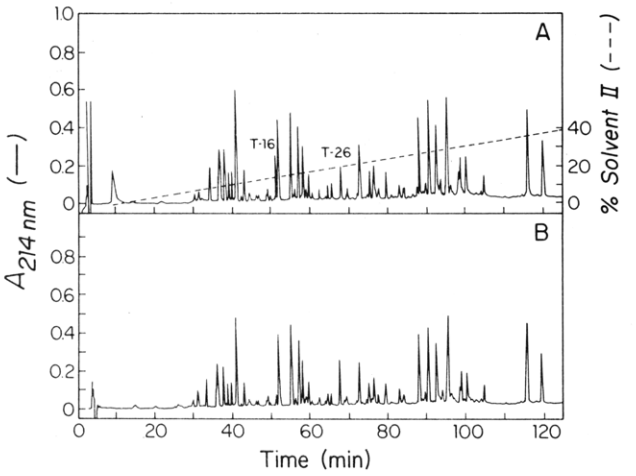

FIGURE 1: Fractionation of [^{3}H]aaNAD$^+$-labeled peptides by reverse-phase HPLC. The peptides were separated on a Vydac C-18 column (25 mm × 5 mm). A 120-min gradient program from 100% solvent I (0.1% TFA) to 40% solvent II (TFA/water/acetonitrile 0.1:9.9:90, v/v/v) at a flow rate of 0.8 mL/min was used. Fractions were manually collected, and 10-μL aliquots from each peak fraction were counted for ^{3}H radioactivity. (A) [^{3}H]aaNAD$^+$-labeled preparation; (B) control.

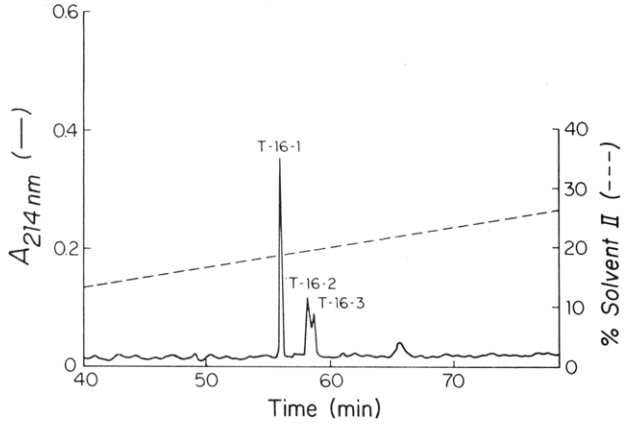

FIGURE 2: Rechromatography of [^{3}H]aaNAD$^+$-labeled peptide. The [^{3}H]aaNAD$^+$-labeled T-16 fraction from reverse-phase HPLC on the Vydac C-18 column (Figure 1A) was further purified on an Altex Ultrasphere ODS column.

of the aaNAD$^+$-labeled peptide. Rechromatography of fraction T-16 on an Altex Ultrasphere ODS column resulted in three peaks (Figure 2). Of the three peaks, T-16-1 was the only one that was radioactive.

Table I: Sequence Analysis of Arylazido-β-alanyl-NAD$^+$-Modified Peptide[a]

cycle	amino acid released	pmol found	radioact. found (cpm)
1	Ile	20	525
2	Val	50	45
3	Ser	112	24
4	Asn	280	15
5	Ala	160	3
6	Ser	68	0
7	X		273
8	Thr	62	96
9	Thr	52	63
10	Asn	20	21

[a] Approximately 400 pmol of peptide was subjected to sequence analysis.

Table II: Amino Acid Composition of Arylazido-β-alanyl-NAD$^+$-Modified Peptide

amino acid	mol/mol	amino acid	mol/mol
asparagine	2 (2)	valine[a]	0.4 (1)
threonine	2.4 (2)	isoleucine[a]	0.3 (1)
serine	2.4 (2)	cysteine[b]	0.8 (1)
alanine	1.1 (1)		

[a] A low recovery of hydrophobic amino acid residues occurring in a cluster is typical. [b] Determined as cysteic acid.

Automated Edman microsequence analysis of the peptide gave the sequence Ile-Val-Ser-Asn-Ala-Ser-X-Thr-Thr-Asn (Table I) where X indicates a cycle for which no call was made. This sequence is identical (except for the blank cycle) with the region from Ile-143 to Asn-152 in pig muscle glyceraldehyde-3-phosphate dehydrogenase (Harris & Perham, 1968). The region is completely conserved in the same enzymes from five species (Walker et al., 1980). By homology, the amino acid at the seventh position should be cysteine, which is not easily detected without derivatization. Cysteine was evident in the amino acid composition analysis (Table II), while no unusual derivatives were detected. The distribution of radioactive label among individual amino acids was determined by counting one-third of the PTH-amino acid derivatives obtained from microsequencing. Significant amounts of radioactivity were found in cycles 1 and 7 (Table I).

In the FAB mass spectrum of the modified peptide (Figure 3), the prominent ion evident at m/z 1232.4 was assigned as the protonated molecular ion. This mass is consistent with the sequence having cysteine at position 7 and the aryl-β-alanyl portion of the radioactive label (calculated monoisotopic mass for the protonated molecular ion = 1232.5). Arylazido-β-

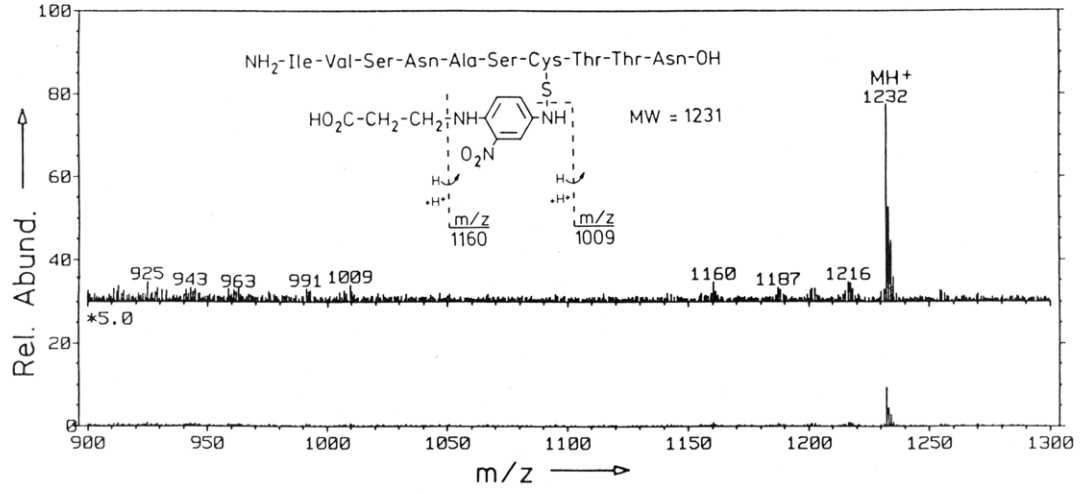

FIGURE 3: Positive ion FAB mass spectrum and proposed structure of the aaNAD$^+$-labeled peptide.

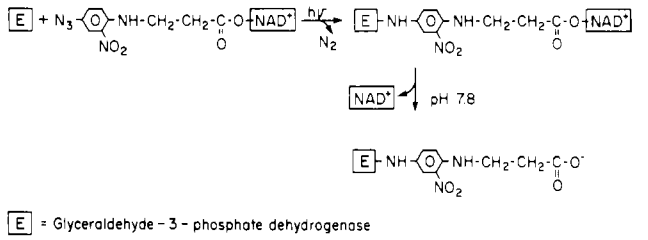

FIGURE 4: Covalent modification of glyceraldehyde-3-phosphate dehydrogenase.

alanyl-NAD^+ was synthesized by coupling the photoreactive group arylazido-β-alanine to NAD^+ through formation of an ester linkage between the carboxyl group of arylazido-β-alanine and the 2′- or 3′-hydroxyl group of the ribose moiety of the AMP portion of the NAD^+ molecule (Chen & Guillory, 1977). Because this ester linkage is not stable under alkali conditions (Jeng & Guillory, 1975), the NAD^+ moiety of the photoprobe would be expected to be lost during the overnight trypsin digestion at pH 7.8 at 37 °C (Figure 4) and would account for the peak that eluted between 8 and 11 min in the chromatogram of the aaNAD^+-modified sample (Figure 1A).

A sulfenamide structure is proposed for the modified peptide (Figure 3). Although a number of fragment ions in the mass spectrum are consistent with loss of various parts of the labeling group, two in particular (m/z 1009 and 1160) are not readily rationalized as coming from the peptide portion of the molecule (see Figure 3 for assignments). The ion at m/z 1009 corresponds to the protonated molecular ion of the underivatized peptide. With successive scans, the intensity of the m/z 1009 ion increases with respect to the protonated molecular ion. In the presence of the dithiothreitol/dithioerythritol matrix, it is likely that the derivatized peptide is slowly converted to the free thiol. Additionally, when a second portion of the modified peptide was analyzed 2 days later, the relative intensity of the ion at m/z 1009 was >20% of the MH^+ ion at 1232, indicating that some of the label was lost during storage.

The labile nature of the modifying group provides a ready explanation for the significant amount of radioactivity found in the first cycle of the microsequencing run. Partial release of the modifying group during the interval between isolation and the first cycle of Edman degradation would result in recovery of radioactivity with the first PTH derivative. Similar results were obtained in the analysis of a photoreactive phospholipid probe modified peptide in the subunit of *Escherichia coli* ATP synthase by Hoppe et al. (1983). It is unlikely that a peptide modified at Ile and a peptide modified at Cys would have the same HPLC retention time. Assuming that the attachment of the label proceeds through a nitrene intermediate, interaction with the side chain of Ile would result in the formation of a carbon–nitrogen bond producing a stable derivative [see review by Bayley & Staros (1984)]. A normal Ile PTH derivative was observed at cycle one. However, the possibility that some of the peptides are modified at the first position cannot be completely eliminated because the yield of PTH-Ile at the first cycle is indeed very low, and modified PTH-Ile may go undetected or may be lost during a wash cycle. On the other hand, the yield to PTH-Val at the second cycle was also low, and no radioactivity was found in this cycle (Table I). The low yield for the first two hydrophobic residues in the modified peptide might be due to a mechanical malfunction during the sequence analysis. The problem has been corrected recently. The recovery of the radioactivity after sequence analysis is low (24%), with approximately 10% of the applied radioactivity remaining with the filter disk. The rest of the radioactive labeling is assumed to be lost during the wash.

Trypsin cleavage after Asn rather than Arg or Lys is unusual. It would appear that cleavage after Asn is a result of modification of the protein by aaNAD^+ because no evidence for such a cleavage was found for the unmodified protein. Careful examination of the HPLC chromatograms of the tryptic digests of the normal and modified enzymes revealed on peak (T-26, Figure 1) which was significantly less intense in the modified protein chromatogram. FAB/MS analysis revealed a molecular ion at m/z 1704, corresponding to the expected tryptic peptide Ile-142 to Lys-159 with a disulfide linkage between Cys-149 and Cys-153. The identity of this peptide was further confirmed by microsequencing analysis (results not shown). Thus, the unmodified tryptic peptide has 17 residues and ends in Lys, whereas the modified tryptic peptide ends in Asn. In addition, a molecular ion at m/z 715, which is consistent with the expected mass of peptide containing residues Cys-153 to Lys-159, was found in the FAB spectrum of the tryptic digest mixture of the modified protein, but not in that of the control sample.

The amount of T-26 in the labeled enzyme tryptic digest is approximately 75% of that in the control mixture (see Figure 1), as would be expected because aaNAD^+ binds to the enzyme in a negative cooperative fashion as does the natural cofactor NAD^+. It has been previously shown that a complete inhibition of the enzyme is associated with only 1 mol of analogue bound per mole of enzyme (i.e., 1 mol of analogue/4 mol of subunits) (Chen et al., 1984).

The present study clearly indicates that aaNAD^+ labels rabbit muscle glyceraldehyde-3-phosphate dehydrogenase at Cys-149 or an amino acid residue near it. This region (Ile-143 to Asn-152) is highly conserved in different species and is part of the active site of the enzyme by X-ray diffraction studies (Moras et al., 1975; Biesacker, 1977). A recent investigation of the aaNAD^+ labeling site on bovine heart β-hydroxybutyrate dehydrogenase also found that a cysteine residue is the site of modification (Yamaguchi et al., 1986).

Although it is expected that the functional groups of all amino acids can react with arylnitrenes or intermediates derived from them (Bayley & Staros, 1984), only three amino acid residues have been identified so far to be labeled in arylazido probe modified peptides: tyrosine (it is found in most investigations, e.g., Lifter et al., 1974; Kerlavage & Taylor, 1980; Knight & McEntee, 1985), cysteine (Hoppe et al., 1983), and proline residues (Bubis & Taylor, 1985). In the present study, we found that aaNAD^+ labels specifically the cysteine residue (Cys-149) although both tyrosine (Tyr-317; it is very near Cys-149) and proline residues (Pro-188, Pro-33, and Pro-79) are present in the active site of glyceraldehyde-3-phosphate dehydrogenase [see Moras et al. (1975)]. This specificity may be due to the fact that Cys-149 functions as a reactive nucleophile involved in enzymatic catalysis as suggested by Rossmann et al. (1977), and may be preferentially attacked by the electrophilic arylnitrenes. Because the Cys-149 is situated at the nicotinamide binding region in the active site of glyceraldehyde-3-phosphate dehydrogenase, it would appear that the arylazido-β-alanyl group of aaNAD^+ must point toward the nicotinamide ring of the NAD^+ even though the group is linked to the ribose moiety of the AMP portion of the molecule [see Chen & Guillory (1977)]. The present finding is important because it indicates that aaNAD^+ may bind to the nicotinamide subsite in the active site of NAD(H)-dependent dehydrogenases. Such a conclusion is also suggested from other studies of aaNAD^+ labeling. Except

for yeast alcohol dehydrogenase, which is an enzyme with A-side specificity (Chen & Guillory, 1977), it has been found so far that all the known enzymes photolabeled by aaNAD+ are those with B-side specificity: mitochondrial NADH dehydrogenase (Chen & Guillory, 1981), mitochondrial NADH–NAD+ transhydrogenase (Chen & Guillory, 1984a), β-hydroxybutyrate dehydrogenase (Yamaguchi et al., 1985), and glyceraldehyde-3-phosphate dehydrogenase (Chen et al., 1984). These results suggest that the arylazido group of aaNAD+ may be situated near the nicotinamide group in such a way that it keeps the latter group in a preferred syn conformation, the conformation imposed when NAD+ binds to enzymes with a B-side specificity. Arylazido-β-alanyl-NAD+ has been shown to be a substrate or competitive inhibitor for a couple of A-side-specific enzymes: mitochondrial NADPH–NAD+ transhydrogenase (Chen & Guillory, 1984b) and microsomal NADH–cytochrome b_5 reductase (S. Chen, M. Haniu, T. Iyanagi, and J. E. Shively, unpublished observation). However, photodependent inactivation of these latter enzymes by aaNAD+ could not be demonstrated, suggesting that aaNAD+ may bind to the A-side-specific enzyme in a conformation that prevents effective covalent incorporation.

In conclusion, we have identified the aaNAD+-modified site in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by direct gas phase microsequencing and FAB mass spectral analyses of the photoprobe-modified peptide. Arylazido-β-alanyl-NAD+ labels the enzyme specifically at the Cys-149 that is at the nicotinamide binding region of the active site. These results indicate that aaNAD+ will be a very useful NAD+ photoaffinity analogue to characterize the active sites of NAD(H)-dependent enzymes.

Registry No. aaNAD+, 64700-08-9; NAD+, 53-84-9; L-Cys, 52-90-4; glyceraldehyde-3-phosphate dehydrogenase, 9001-50-7.

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