O'Sullivan, W. J. (1969) Stability constants of metal complexes, in *Data for biochemical research* (Dawson, R. M. C., Elliot, D. C., Elliot, W. H., & Jones, K. M., Eds.) pp 423-434, Oxford University Press, London.
Pannbacker, R. G. (1973) *Science 182*, 1138-1140.
Paul, A. K., Marala, R. B., Jaiswal, R. K., & Sharma, R. K. (1987) *Science 235*, 1224-1226.
Pepe, I. M., Boero, A., Vergani, L., Panfoli, I., & Cugnoli, C. (1986) *Biochim. Biophys. Acta 889*, 271-276.
Pugh, E. N., Jr., & Cobbs, W. H. (1986) *Vision Res. 26*, 1613-1643.
Ratto, G. M., Payne, R., Owen, W. G., & Tsien, R. Y. (1988) *J. Neurosci. 8*, 3240-3246.
Read, S. M., & Northcote, D. H. (1981) *Anal. Biochem. 116*, 53-64.
Ridlington, J. W., & Butler, L. G. (1972) *J. Biol. Chem. 247*, 7303-7307.
Schleicher, A., Kuhn, H., & Hofmann, K. P. (1989) *Biochemistry 28*, 1770-1775.
Schnetkamp, P. P. M., & Kaupp, U. B. (1985) *Biochemistry 24*, 723-727.
Sitaramayya, A. (1986) *Biochemistry 25*, 5460-5468.
Sitaramayya, A., & Liebman, P. A. (1983) *J. Biol. Chem. 258*, 12106-12109.
Springs, B., Welsh, K. M., & Coperman, B. S. (1981) *Biochemistry 20*, 6384-6391.
Stryer, L. (1986) *Annu. Rev. Neurosci. 9*, 87-119.
Tremblay, J., Gerzer, R., Pang, S. C., Cantin, M., & Hamet, P. (1986) *FEBS Lett. 194*, 210-214.
Tsien, R. Y. (1980) *Biochemistry 19*, 2396-2404.
Wilden, U., Hall, S. W., & Kuhn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174-1178.
Zuckerman, R., & Chaesty, J. E. (1986) *FEBS Lett. 207*, 35-41.

N-Arylazido-β-alanyl-NAD$^+$, a New NAD$^+$ Photoaffinity Analogue. Synthesis and Labeling of Mitochondrial NADH Dehydrogenase†

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ABSTRACT: N-Arylazido-β-alanyl-NAD$^+$ [N3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$] has been prepared by alkaline phosphatase treatment of arylazido-β-alanyl-NADP$^+$ [N3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NADP$^+$]. This NAD$^+$ analogue was found to be a potent competitive inhibitor (K_i = 1.45 μM) with respect to NADH for the purified bovine heart mitochondrial NADH dehydrogenase (EC 1.6.99.3). The enzyme was irreversibly inhibited as well as covalently labeled by this analogue upon photoirradiation. A stoichiometry of 1.15 mol of N-arylazido-β-alanyl-NAD$^+$ bound/mol of enzyme, at 100% inactivation, was determined from incorporation studies using tritium-labeled analogue. Among the three subunits, 0.85 mol of the analogue was bound to the M_r = 51 000 subunit, and each of the two smaller subunits contained 0.15 mol of the analogue when the dehydrogenase was completely inhibited upon photolysis. Both the irreversible inactivation and the covalent incorporation could be prevented by the presence of NADH during photolysis. These results indicate that N-arylazido-β-alanyl-NAD$^+$ is an active-site-directed photoaffinity label for the mitochondrial NADH dehydrogenase, and are further evidence that the M_r = 51 000 subunit contains the NADH binding site. Previous studies using A-arylazido-β-alanyl-NAD$^+$ [A3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$] demonstrated that the NADH binding site is on the M_r = 51 000 subunit [Chen, S., & Guillory, R. J. (1981) *J. Biol. Chem. 256*, 8318-8323]. Results are also presented to show that N-arylazido-β-alanyl-NAD$^+$ binds the dehydrogenase in a more effective manner than A-arylazido-β-alanyl-NAD$^+$.

The mitochondrial NADH dehydrogenase (EC 1.6.99.3) is the first enzyme of the respiratory chain, which conveys the electrons derived from NADH to ubiquinone via FMN and eight or nine iron–sulfur clusters as redox components (Ohnishi et al., 1985; Hatefi, 1985; Ragan, 1987). Upon treatment of the membrane-bound form of the enzyme, complex I, with chaotropic reagents (Davis & Hatefi, 1969; Galante & Hatefi, 1979), a water-soluble iron–sulfur flavoprotein preparation of NADH dehydrogenase can be isolated. This soluble enzyme preparation contains three subunits with molecular masses of 51 000, 24 000, and 9000 daltons and is capable of oxidizing NADH in the presence of quinones or ferric complexes as electron acceptors (Galante & Hatefi, 1979). Chen and Guillory (1981) showed that the M_r = 51 00 subunit of this enzyme could be labeled with tritiated A-arylazido-β-alanyl-NAD$^+$[1] (it was previously named arylazido-β-alanyl-NAD$^+$, further details under Results and Discussion), suggesting that the NADH binding site is on this subunit.

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[1] Abbreviations: N-arylazido-β-alanyl-NAD$^+$, N3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$; A-arylazido-β-alanyl-NAD$^+$, A3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$; arylazido-β-alanyl-NADP$^+$, N3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl}NADP$^+$; SDS, sodium dodecyl sulfate.

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The synthesis of A-arylazido-β-alanyl-NAD+ was reported in 1977 (Chen & Guillory, 1977), and it has been shown to be a NAD+ photoaffinity label for yeast alcohol dehydrogenase (Chen & Guillory, 1977), yeast glyceraldehyde-3-phosphate dehydrogenase (Bayne et al., 1981), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Chen et al., 1984), bovine heart β-hydroxybutyrate dehydrogenase (Yamaguchi et al., 1985), and the mitochondrial NADH–NAD+ transhydrogenase (Chen & Guillory, 1979, 1984) besides the mitochondrial NADH dehydrogenase. A-Arylazido-β-alanyl-NAD+ was found to label the essential Cys-149 in the active site of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Chen et al., 1986). The same analogue also modified a Cys residue in β-hydroxybutyrate dehydrogenase (Yamaguchi et al., 1986). Since the modified peptide of the bovine heart β-hydroxybutyrate dehydrogenase has a homologous amino acid sequence to that found in the rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, it was suggested that the peptide represents a portion of the active site of β-hydroxybutyrate dehydrogenase. These studies indicate that A-arylazido-β-alanyl-NAD+ is a useful photoaffinity NAD+ analogue for investigating the NAD(H) binding site of NAD(H)-dependent enzymes. The site modified by A-arylazido-β-alanyl-NAD+ in the mitochondrial NADH dehydrogenase is not yet determined.

In this paper, we describe the preparation of a photoaffinity analogue of NAD+, N-arylazido-β-alanyl-NAD+, by alkaline phosphatase treatment of arylazido-β-alanyl NADP+. The resulting analogue has the arylazido-β-alanyl group attached to the ribose in the NMN portion of the NAD+ molecule in contrast to A-arylazido-β-alanyl NAD+ (Chen & Guillory, 1977) which has the arylazido-β-alanyl group attached to the ribose of the adenine portion of the NAD+ molecule. Results are presented to show that this newly synthesized analogue is a more effective photodependent active-site-directed label of the mitochondrial NADH dehydrogenase than A-arylazido-β-alanyl NAD+. The new analogue also labeled the M_r = 51 000 subunit, but in a much more selective manner.

Materials and Methods

Reagents and Enzyme Preparations. The reagents NADP+ and NADH were obtained from Sigma. Arylazido-β-alanyl-NADP+ and its radioactive derivative were synthesized as described previously by Chen and Guillory (1980). A-Arylazido-β-alanyl-NAD+ was synthesized according to the published procedure (Chen & Guillory, 1977).

The mitochondrial NADH dehydrogenase was resolved from complex I by $NaClO_4$ treatment and ammonium sulfate fractionation according to the procedure described by Galante and Hatefi (1979). Complex I was isolated from bovine heart mitochondria according to the procedure of Hatefi et al. (1962). Alkaline phosphatase used in this study was obtained from Sigma.

The dehydrogenase was assayed by using NADH as the electron donor and potassium ferricyanide as the electron acceptor by a procedure previously described (Chen & Guillory, 1985). The enzymatic assays were always performed in duplicate, and good agreement was always found between the two measurements. The specific activity of this enzyme preparation was 185 μmol of potassium ferricyanide reduced min^{-1} (mg of protein)$^{-1}$.

Synthesis of N-Arylazido-β-alanyl-NAD+. N-Arylazido-β-alanyl-NAD+ was prepared by removing the 2′-phosphate of arylazido-β-alanyl-NADP+ with alkaline phosphatase treatment. The detailed procedure is described under Results and Discussion.

Photolysis. The photolytic conditions are those previously described (Chen & Guillory, 1977).

Determination of the Stoichiometry of N-Arylazido-β-alanyl-NAD+ Labeling. NADH dehydrogenase (150 μg/mL) was photoirradiated for 1 min in 50 mM potassium phosphate buffer, pH 7.4, containing N-arylazido-β-[3-^{3}H]alanyl-NAD+ (specific activity of 2.8×10^7 cpm/μmol) from 0 to 40 μM. The photoirradiation was done at 30-s intervals to prevent overheating. A control reaction, performed in the absence of N-arylazido-β-alanyl-NAD+, indicated that the enzyme activity was not affected by this procedure. After photoirradiation, 2-μL aliquots were withdrawn and assayed for residual activity as described above. The measured activity immediately prior to photoirradiation was taken as 100%. No significant inhibition was observed for all samples when the assay was performed prior to irradiation, indicating that the small amount of N-arylazido-β-alanyl-NAD+ present during the assay had no effect on activity. The rest of the sample was denatured in the presence of 1% sodium dodecyl sulfate (SDS) by incubation in a boiling water bath for 2 min. The labeled enzymes were then analyzed by SDS–polyacrylamide gel electrophoresis according to the procedure described by Laemmli (1970). The Coomassie blue stained protein bands were sliced from the gel, and the gel pieces were dissolved in 0.2 mL of 30% hydrogen peroxide. The bound radioactivity was then evaluated.

Results and Discussion

Preparation and Characterization of N-Arylazido-β-alanyl-NAD+. N-Arylazido-β-alanyl-NAD+ was prepared by alkaline phosphatase treatment of arylazido-β-alanyl-NADP+ to remove the 2′-phosphate group, followed by thin-layer chromatographic separation. Details are as follows. Upon incubation of 10 mM arylazido-β-alanyl-NADP+ (in 1 mL of 0.1 M NaHepes buffer, pH 8.0) with 10 units of *Escherichia coli* alkaline phosphatase for 10 min at 25 °C, all the NADP+ analogue was converted to the NAD+ derivative as shown by thin-layer chromatography on cellulose plates. In the solvent system 1-butanol/water/acetic acid (5/3/2), the product had an R_f value identical with that of A-arylazido-β-alanyl-NAD+. Since arylazido-β-alanyl-NADP+ has one arylazido-β-alanyl group attached to the ribose of the NMN portion of the NADP+ molecule through an ester linkage (Chen & Guillory, 1980), and alkaline phosphatase treatment should not affect the arylazido-β-alanyl group attachment, the new NAD+ analogue should have the arylazido-β-alanyl group at the same position. Therefore, we named this analogue N-arylazido-β-alanyl NAD+ (for structure see Figure 1) to indicate that the arylazido-β-alanyl group is attached to the ribose of the NMN portion of the NAD+ molecule. The arylazido NAD+ analogue reported previously (Chen & Guillory, 1977) was then named A-arylazido-β-alanyl-NAD+ because it has the arylazido-β-ananyl group attached to the ribose of the AMP portion of the NAD+ molecule. The structure of the new NAD+ analogue was confirmed by incubating the compound in 50 mM ammonium bicarbonate, pH 9, for 3 h to hydrolyze the ester linkage. The nucleotide portion of the analogue was separated from arylazido-β-alanine by thin-layer chromatography on cellulose plates using the solvent system 1-butanol/water/acetic acid (5/3/2). It was shown that the nucleotide (R_f = 0.12) was NAD+, not NADP+, because it could be reduced by yeast alcohol dehydrogenase as indicated by the generation of a 340-nm absorption peak in the presence of ethanol. Furthermore, upon treatment of the compound with nucleotide pyrophosphatase from *Crotalus adamanteus* venom (Sigma Chemical Co. type

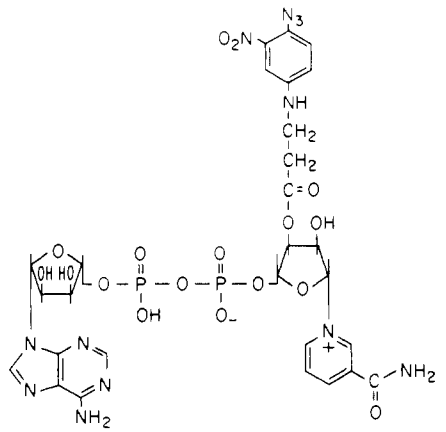

FIGURE 1: Structure of N-arylazido-β-alanyl-NAD$^+$.

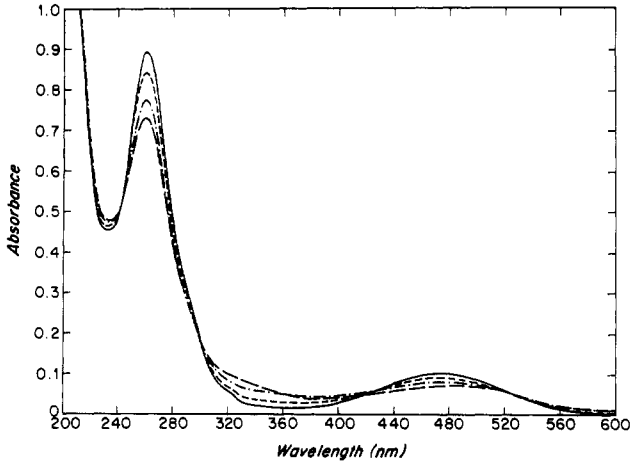

FIGURE 2: Absorption spectrum of N-arylazido-β-alanyl-NAD$^+$ and the spectral changes upon photoirradiation. The absorption spectra of N-arylazido-β-alanyl-NAD$^+$ (2.51×10^{-5} M) in 1 mL of distilled water upon photoirradiation ion for 0 (—), 1 (- - -), 2 (-·-), and 4 min (– –).

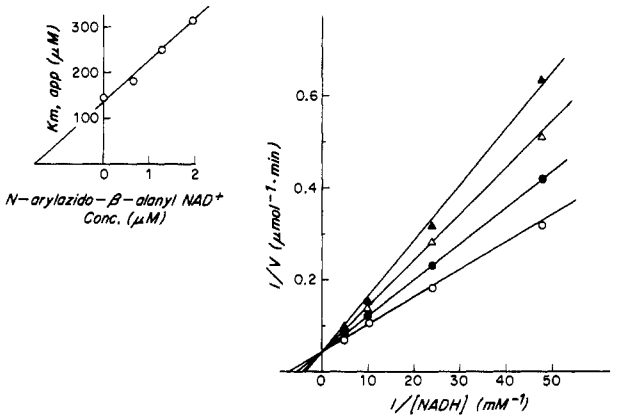

FIGURE 3: Competitive inhibition of NADH dehydrogenase by N-arylazido-β-alanyl-NAD$^+$. The NAD$^+$ analogue concentrations of 0 (○), 0.65 (●), 1.3 (△), and 2.0 μM (▲). K_i determination is shown in the upper left plot.

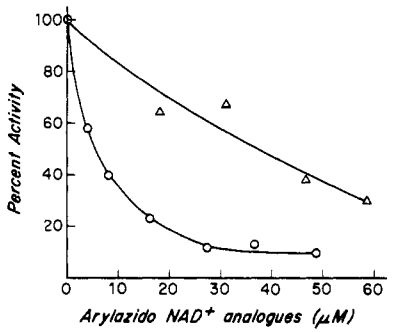

FIGURE 4: Photodependent inhibition of NADH dehydrogenase by N-arylazido-β-alanyl-NAD$^+$ (○) or A-arylazido-β-alanyl-NAD$^+$ (△). NADH dehydrogenase (30 μg) was incubated with N-arylazido-β-alanyl-NAD$^+$ or A-arylazido-β-alanyl-NAD$^+$ at the indicated concentrations in 200 μL of sodium phosphate buffer, pH 7.4, and irradiated for 1 min. The enzyme activity before irradiation was taken as 100%. The experimental procedures are those described under Materials and Methods.

II), two products were formed. these two products were separated by thin-layer chromatography and identified as arylazido-β-alanyl-NMN and AMP, respectively. We did not determine whether the arylazido-β-alanyl group interacts with the 2′- or 3′-hydroxy of the ribose in N-arylazido-β-alanyl-NAD$^+$, but it was previously suggested that the 3′-hydroxyl group is involved in esterified for arylazido-β-alanyl-NADP$^+$ (Guillory et al., 1980). Figure 2 shows the absorption spectrum of N-arylazido-β-alanyl-NAD$^+$ and the spectral changes upon photoirradiation. The ratio of the absorbance of the compound without irradiation at 260 nm (molar extinction coefficient of 35.6×10^3) and 475 nm (molar extinction coefficient of 4.7×10^3) is 1:0.13, which is identical with that of A-arylazido-β-alanyl-NAD$^+$, confirming a 1:1 stoichiometry of NAD$^+$ and arylazido-β-alanine in this analogue. The photodependent conversion of the azido group of the nucleotide analogue to a nitrene species was demonstrated by the typical photodependent spectral changes. N-Arylazido-β-alanyl-NAD$^+$ is, however, different from A-arylazido-β-alanyl NAD$^+$ in that it is not a substrate of yeast alcohol dehydrogenase.

Inhibition of the Mitochondrial NADH Dehydrogenase by N-Arylazido-β-alanyl-NAD$^+$. As expected, without photoirradiation, N-arylazido-β-alanyl-NAD$^+$ is a competitive inhibitor with respect to NADH for the mitochondrial NADH dehydrogenase (Figure 3). A K_i value of 1.45 μM was calculated, and it is very similar to that determined for A-arylazido-β-alanyl-NAD$^+$, 1.2 μM (Chen & Guillory, 1981). These results indicate that both of these NAD$^+$ analogues are potent inhibitors of the mitochondrial NADH dehydrogenase and suggest that they bind to the enzyme at the active site with approximately equal affinity. The position of the arylazido-β-alanyl group seems not be affect the interaction of these NAD$^+$ analogues with the enzyme.

In contrast to the finding that in the dark the two NAD$^+$ analogues have similar binding affinity toward the mitochondrial NADH dehydrogenase, upon photoirradiation, N-arylazido-β-alanyl-NAD$^+$ inhibited the enzyme much more effectively than A-arylazido-β-alanyl NAD$^+$. Following a 1-min irradiation in the presence of 5 μM N-arylazido-β-alanyl-NAD$^+$, the NADH–ferricyanide reductase activity of the NADH dehydrogenase was inhibited 50% as estimated from the inhibitory curve shown in Figure 4, while 35 μM A-arylazido-β-alanyl-NAD$^+$ would be required to inhibit the enzyme 50% under the same photolytic conditions. These results indicate that the position of the arylazido-β-alanyl group has a definite influence on the photodependent incorporation of these analogues at the active site of the mitochondrial NADH dehydrogenase. It is possible that the ribose of the AMP portion of the NAD(H) molecule may be in a more exposed environment when NAD(H) binds to the active site of the mitochondrial NADH dehydrogenase. Therefore, upon irradiation, A-arylazido-β-alanyl-NAD$^+$ incorporates into the active site of the enzyme with a lower efficiency. Irradiation in the presence of 27 μM N-arylazido-β-alanyl-NAD$^+$ resulted in 89% inhibition. Irradiation in the presence of higher concentrations of the N-arylazido-β-alanyl-NAD$^+$ did not result

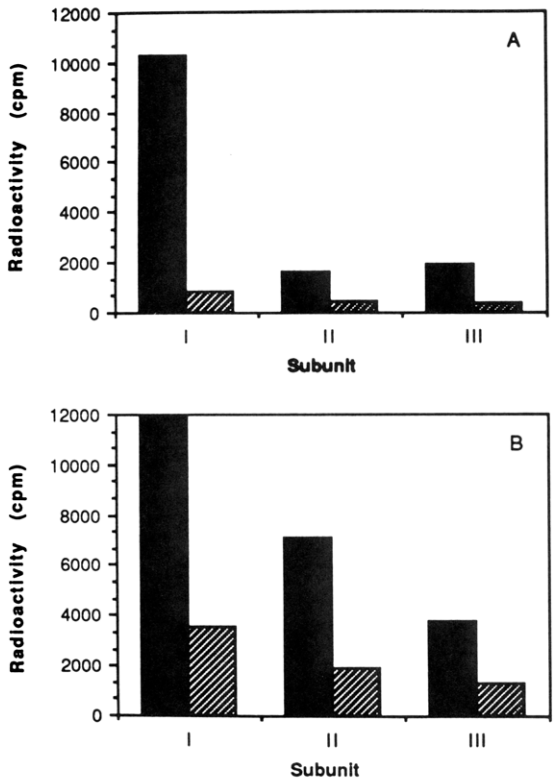

FIGURE 5: Photodependent arylazido-β-[3-^{3}H]alanyl-NAD+ labeling of NADH dehydrogenase. NADH dehydrogenase (180 μg/1.2 mL) was photolyzed for 1 min in the presence of 24 nmol of N-arylazido-β-[3-^{3}H]alanyl-NAD+ (2.2×10^7 cpm/μmol) (A) or 77 nmol of A-arylazido-β-[3-^{3}H]alanyl-NAD+ (4.2×10^7 cpm/μmol) (B). The labeled enzymes were subjected to SDS gel electrophoresis. The protein bands were dissolved in 30% hydrogen peroxide and counted for radioactivity. Subunits I, II, and III are M_r = 51 000, 24 000, and 9000 subunits, respectively. (Solid block) Arylazido NAD+ analogue labeling in the absence of NADH; (hatched block) arylazido NAD+ analogue labeling in the presence of NADH.

in much more inhibition. It is not unusual that a 100% inhibition is hard to obtaiun in experiments with photoaffinity probes, and the nature of this phenomenon has not been completely understood. One possible explanation is that the unbound photoproducts interfere with the specific insertion of the analogue. It is worth mentioning that it was previously shown that no inhibition of the enzymatic activity was observed when the enzyme was photoirradiated in the presence of either 91 μM arylazido-β-alanine, 75 μM NAD+, or 367 μM arylazido-β-alanyl-NADP+ (Chen & Guillory, 1981), indicating that the observed inhibition by the NAD+ analogues is due to specific interaction of arylazido-NAD+ derivatives with the mitochondrial NADH dehydrogenase. Since the enzyme assay was performed with only 2 μL of the irradiation mixture, the final concentration of N-arylazido-β-alanyl-NAD+ in the assay was $^1/_{500}$ of that during the irradiation (the volume of the assay solution is 1 mL). NAD+ analogue concentrations up to 0.1 μM did not inhibit the enzyme significantly when added directly during the enzyme assay. The photodependent inhibition by N-arylazido-β-alanyl-NAD+ could be prevented by the presence of NADH in the irradiation mixture, suggesting that N-arylazido-β-alanyl-NAD+ modification occurs at the NADH binding site of the NADH dehydrogenase. Enzyme inactivation was effectively prevented with a NADH concentration 12-fold in excess over that of N-arylazido-β-alanyl-NAD+.

Photodependent Labeling of the Mitochondrial NADH Dehydrogenase. As indicated previously, the purified NADH dehydrogenase consists of three subunits, M_r = 51 000, 24 000,

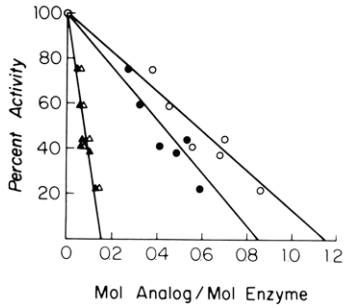

FIGURE 6: Correlation of enzyme inhibition and extent of labeling of NADH dehydrogenase by N-arylazido-β-alanyl NAD+. Conditions are those described in Figure 5 except the analogue concentrations were different. (○) Total labeling; (●), labeling associated with the M_r = 51 000 subunit; (△) labeling associated with the M_r = 24 000 subunit; (▲), labeling associated with the M_r = 9000 subunit.

and 9000. When 180 μg of the dehydrogenase was photoirradiated for 1 min in 1.2 mL of solution containing 50 mM phosphate buffer, pH 7.4, and 24 nmol of N-arylazido-β-[3-^{3}H]alanyl-NAD+, most of the radioactive label was found to be associated with the M_r = 51 000 subunit (Figure 5A). This result confirms that the NADH binding site is on the M_r = 51 000 subunit. Under the specific conditions of this experiment, the NADH–ferricyanide reductase activity of the dehydrogenase was inhibited 57%. The radioactive labeling was greatly reduced when labeling was performed in the presence of 0.5 mM NADH, confirming that the NAD+ analogue binds to the active site of the enzyme. As a comparison, when the enzyme was photolyzed under the same conditions in the presence of 77 nmol of A-arylazido-β-[3-^{3}H]alanyl-NAD+, the M_r = 51 000 subunit was the major subunit labeled by the analogue. However, significant amounts of radioactive label were associated with the M_r = 24 000 and 9000 subunits (Figure 5B). This amount of A-arylazido-β-alanyl-NAD+ was chosen for producing a similar degree of inactivation of the enzyme. The enzyme activity was found to be inhibited 65% in the latter sample. The radioactive labeling was also reduced when labeling was performed in the presence of 3 mM NADH. These results indicate that N-arylazido-β-alanyl-NAD+ labels the NADH dehydrogenase in a more selective manner than A-arylazido-β-alanyl NAD+. It was previously shown that addition of arylazido-β-alanine during irradiation of the dehydrogenase with A-arylazido-β-alanyl-NAD+ could reduce the background of the labeling (Chen & Guillory, 1981). However, since the nucleotide analogue labeling associated with the M_r = 24 000 and 9000 subunits could also be mostly protected by NADH, it is possible that the NADH binding site on the 51K subunit is near the region where this subunit interacts with the two smaller subunits (further discussion later).

Using a molecular weight of 84 000 (51 000 + 24 000 + 9000), we determined that a 100% inhibition of the NADH dehydrogenase would be observed at 1.15 mol of N-arylazido-β-alanyl-NAD+ bound/mol of dehydrogenase (Figure 6). This analysis indicates that the analogue binds to the enzyme in a stoichiometric and specific manner. There would be 0.85 mol of analogue bound/mol of the M_r = 51 000 subunit at 100% inhibition of the activity, confirming again that the NADH binding site is on the M_r = 51 000 subunit. It is interesting to find that approximately 0.15 mol of the analogue bound/mol of the M_r = 24 000 or 9000 subunit when the enzyme was inhibited 100%. Figure 7 shows that the N-arylazido-β-alanyl-NAD+ labeling of the M_r = 51 000 subunit could be protected by NADH in a concentration-dependent manner. It is interesting to find that the labeling associated

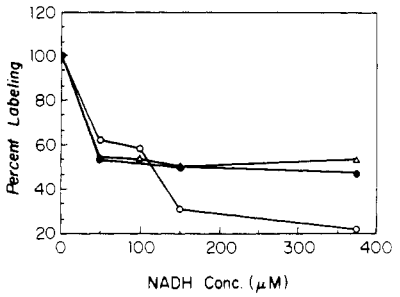

FIGURE 7: Protection by NADH of the binding of N-arylazido-β-alanyl-NAD$^+$ to NADH dehydrogenase. NADH dehydrogenase (30 μg/200 μL) was photolyzed with 5 nmol of N-arylazido-β-[3-^{3}H]-alanyl-NAD$^+$ (2.8×10^7 cpm/μmol) in the presence of NADH at the indicated concentrations. Other details are identical with those described in Figure 6. The amount of the NAD$^+$ analogue associated with each subunit in the absence of NADH was taken as 100%. The labeling associated with the M_r = 51 000, 24 000, or 9000 subunits is indicated as ○, ●, or △, respectively.

with the M_r = 24 000 and 9000 subunits could also be protected by NADH. However, our results seem to suggest that only a portion (50%) of the labeling associated with the two small subunits is related to NADH binding. These results again suggest that the three subunits of the NADH dehydrogenase may interact with each other, and the NADH binding site on the M_r = 51 000 subunit may be near the region involving the attachment of the two smaller subunits.

In conclusion, we have described the synthesis of a photoaffinity analogue of NAD$^+$, N-arylazido-β-alanyl-NAD$^+$. We have presented results demonstrating that N-arylazido-β-alanyl-NAD$^+$ is an active-site-directed reagent for the mitochondrial NADH dehydrogenase. This NAD$^+$ analogue is a potent competitive inhibitor with respect to NADH in the dark (Figure 3), and a photodependent inhibition of the enzyme was demonstrated (Figure 4). A stoichiometry of 1 mol of the analogue bound/mol of the enzyme was determined from incorporation studies using radioactive analogue (Figure 6). We found that the irreversible inactivation as well as the covalent incorporation (Figures 5 and 7) could be completely prevented by the presence of NADH during photolysis. Since both N-arylazido-β-alanyl-NAD$^+$ and A-arylazido-β-alanyl-NAD$^+$ can bind to the active site of the mitochondrial NADH dehydrogenase, and these two compounds have the arylazido-β-alanyl group at two different riboses of the NAD$^+$ molecule, they will be useful for investigating the regions in the active site of the NADH dehydrogenase that interact with each ribose and the pyrophosphate group of NAD(H).

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Registry No. N-Arylazido-β-alanyl-NAD$^+$, 124201-58-7; arylzido-β-alanyl-NADP$^+$, 73617-93-3; NADH, 58-68-4; NADH dehydrogenase, 9079-67-8.

REFERENCES

Bayne, S., Sund, H., & Guillory, R. J. (1981) *Biochimie 63*, 569–573.

Chen, S., & Guillory, R. J. (1977) *J. Biol. Chem. 252*, 8990–9001.

Chen, S., & Guillory, R. J. (1979) *J. Biol. Chem. 254*, 7220–7227.

Chen, S., & Guillory, R. J. (1980) *J. Biol. Chem. 255*, 2445–2453.

Chen, S., & Guillory, R. J. (1981) *J. Biol. Chem. 256*, 8318–8323.

Chen, S., Davis, H., Vierra, J. R., & Guillory, R. J. (1984) *Biochem. Biophys. Stud. Proteins Nucleic Acids [Proc. Int. Symp.], 3rd*, 407–425.

Chen, S., & Guillory, R. J. (1984) *J. Biol. Chem. 259*, 5124–5131.

Chen, S., & Guillory, R. J. (1985) *J. Bioenerg. Biomembr. 17*, 33–49.

Chen, S., Lee, T. D., Legesse, K., & Shively, J. E. (1986) *Biochemistry 25*, 5391–5395.

Davis, K. A., & Hatefi, Y. (1969) *Biochemistry 9*, 3355–3361.

Galante, Y. M., & Hatefi, Y. (1979) *Arch. Biochem. Biophys. 192*, 559–568.

Guillory, R. J., Jeng, S. J., & Chen, S. (1980) *Ann. N.Y. Acad. Sci. 346*, 244–279.

Hatefi, Y., Haavik, A. G., & Griffiths, D. E. (1962) *J. Biol. Chem. 237*, 1676–1680.

Hatefi, Y. (1965) *Annu. Rev. Biochem. 54*, 1015–1069.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Ohnishi, T., Ragan, C. I., & Hatefi, Y. (1985) *J. Biol. Chem. 260*, 2782–2788.

Ragan, C. I. (1987) *Curr. Top. Bioenerg. 15*, 1–36.

Yamaguchi, M., Chen, S., & Hatefi, Y. (1985) *Biochemistry 24*, 4912–4916.

Yamaguchi, M., Chen, S., & Hatefi, Y. (1986) *Biochemistry 25*, 4864–4868.