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Photodependent Inhibition of Rat Liver NAD(P)H:Quinone Acceptor Oxidoreductase by (A)-2-Azido-NAD$^+$ and (A)-8-Azido-NAD$^+$†

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ABSTRACT: Two photoaffinity analogues of NAD$^+$, (A)-2-azido-NAD$^+$ [nicotinamide 2-azidoadenine dinucleotide] and (A)-8-azido-NAD$^+$ [nicotinamide 8-azidoadenine dinucleotide], have been synthesized, and their reactivities with the rat liver NAD(P)H:quinone acceptor oxidoreductase have been investigated. The reduce nicotinamide nucleotide probes, (A)-2-azido-NADH and (A)-8-azido-NADH, were shown to be substrates of the quinone reductase. This enzyme was inhibited by (A)-2-azido-NAD$^+$ and (A)-8-azido-NAD$^+$ in a photodependent manner, and the inhibition of the enzyme could be prevented by the presence of nicotinamide nucleotide substrates during photolysis. (A)-2-Azido-NAD$^+$ was demonstrated to be a more potent inhibitor than (A)-8-azido-NAD$^+$. In addition, the photodependent inhibition by (A)-8-azido-NAD$^+$ increased when menadione, the substrate of the enzyme, was present during the photolysis, while menadione protected the enzyme from the photodependent inhibition by (A)-2-azido-NAD$^+$. These results indicate that these two NAD$^+$ analogues can be used to identify the nicotinamide nucleotide binding site of this quinone reductase and that they probably bind to the enzyme in different fashions.

NAD(P)H:quinone acceptor oxidoreductase(EC 1.6.99.2; DT-diaphorase) plays an important role in protecting tissues against the mutagenic, carcinogenic, and cytotoxic effects of quinones that occur widely in nature (Ernster, 1987). Protection is accomplished by the unique property of this quinone reductase to catalyze an obligatory two-electron reduction of several quinones, including vitamin K (e.g., menadione), to hydroquinones with either NADH or NADPH as electron donor (Iyanagi & Yamazaki, 1970; Iyanagi, 1987). This

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reaction bypasses the semiquinone free-radical state and prevents the formation of quinone-mediated oxygen free radicals that are generated by redox cycling of semiquinones in the presence of oxygen.

The primary structure of the rat liver NAD(P)H:quinone acceptor reductase has been deduced from cDNA sequences (Robertson et al., 1986; Bayney et al., 1987) and determined by protein microsequencing methods (Haniu et al., 1988). The amino acid sequence of the human enzyme has also been deduced from the cDNA sequence (Jaswal et al., 1988). This enzyme consists of two equal-size subunits. Each subunit contains 273 residues and one molecule of the FAD prosthetic group (Hosoda et al., 1974; Rase et al., 1976). While the primary structure of this quinone reductase has been determined independently by different groups during the last several years, the structure–function relationship of this enzyme remains unclarified.

This enzme has an unusual nicotinamide nucleotide binding site in that it utilizes either NADH or NADPH as electron donors with equal maximal velocities (Ernster et al., 1962). This indicates that the nicotinamide nucleotide binding site of the enzyme has a structure that can accommodate either NADH or NADPH. We feel that the first step toward understanding the catalytic mechanism of this enzyme is to identify the regions involved in the nicotinamide nucleotide binding. Through affinity-labeling experiments using 5′-[(fluorosulfonyl)benzoyl]adenosine (5′-FSBA),[1] previous studies of the rat liver enzyme from this laboratory revealed a region of this enzyme, ^{146}I-T-T-G-G-S-G-S-M-Y^{155}, that is thought to be a part of the nicotinamide nucleotide binding site of this quinone reductase (Liu et al., 1989). The rat liver NAD(P)H:quinone oxidoreductase has been expressed in COS7 cells, and site-directed mutagenesis experiments near the proposed nicotinamide nucleotide binding site have been initiated (Forrest et al., 1990).

In order to further characterize the nicotinamide nucleotide binding site of NAD(P)H:quinone oxidoreductase, two photoaffinity analogues of NAD^+, (A)-2-azido-NAD^+ and (A)-8-azido-NAD^+, were synthesized in this laboratory. In this report we present results to show that these two NAD^+ analogues were found to be potentially useful photoaffinity labels for this quinone reductase. Some of these results were presented at the FASEB 1990 annual meeting at New Orleans, LA (Deng et al., 1990b).

In addition to these two compounds, four other NAD^+ photoaffinity analogues have also been synthesized and shown not to be useful for the study of the nicotinamide nucleotide binding site of this enzyme. They are (A)-arylazido-β-alanyl-NAD^+, (N)-arylazido-β-alanyl-NAD^+, (A)-(4-benzoylbenzoyl)-NAD^+, and (N)-3-azido-PyAD^+.

Materials and Methods

Enzyme Preparation and Assay. The NAD(P)H:quinone acceptor oxidoreductase was purified from livers of female Wistar rats injected daily for 3 days with 3-methylcholanthrene (4 mg/100 g of body weight), according to a procedure described by Haniu et al. (1988).

The NAD(P)H:quinone acceptor oxidoreductase activity was determined spectrophotometrically by measuring the reduction of cytochrome *c* at 550 nm [ϵ(550 nm) = 18.5×10^3 M^{-1} cm^{-1}] at 25 °C. The assay mixture (1 mL) contains 50 mM sodium phosphate, pH 7.4, 200 μM NADH, 10 μM menadione, and 77 μM cytochrome *c*. In this assay, menadione is the electron acceptor and cytochrome *c* is included in order to continuously reoxidize the menadiol formed. The reaction is initiated with addition of the enzyme. The enzymatic assays are performed in duplicate.

Synthesis of (A)-2-Azido-NAD^+ and (A)-8-Azido-NAD^+. (A)-8-Azido-NAD^+ was synthesized by coupling of 8-azido-AMP with NMN according to the method of Michelson (1964) and purified on a Dowex 1-×2 (formate) column according to the method of Koberstein (1976). 8-Azido-AMP was obtained from Sigma Chemical Co.

The synthesis of (A)-2-azido-NAD^+ involves three steps: conversion of 2-chloroadenosine to 2-azidoadenosine, synthesis of 2-azido-AMP, and coupling of 2-azido-AMP to NMN. 2-Chloroadenosine was converted to 2-azidoadenosine according to the method of Schaefer and Thomas (1958). 2-Azide-AMP was prepared by a method modified from that of Yoshikawa et al. (1969). Briefly, 2-azidoadenosine was reacted with phosphorus oxychloride (freshly distilled) in trimethyl phosphate for 8 h at 0 °C. After extraction with ether to remove trimethyl phosphate, the residue was dissolved in ice water and the pH of the solution was adjusted to pH 7–8 with diluted ammonium hydroxide. 2-Azido-AMP was purified on a DEAE-Sephadex A-25 (bicarbonate form) column with use of a linear gradient of 0–0.5 M triethylammonium bicarbonate (pH 7.8).

(A)-2-Azido-NAD^+ was prepared by coupling of 2-azido-AMP with NMN according to the method of Michelson (1964). The purification of (A)-2-azido-NAD^+ was performed on a Dowex 1-×2 (formate) column with use of a linear gradient of 0–1 M formic acid.

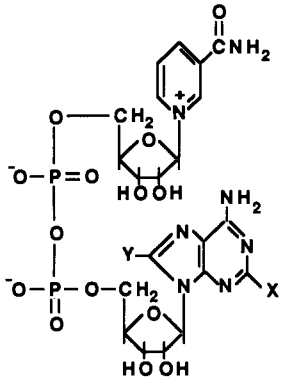
(A)-2-Azido-NAD^+ has also been synthesized recently by Kim and Haley (1990) to study the active site of the bovine liver glutamate dehydrogenase and by Vaillancourt et al. (1990) to study G-protein structure.

Photolysis. The photolytic conditions are those previously described (1977). The quinone reductase (23 μg) was irradiated for 1 or 2 min in 150 μL of 50 mM sodium phosphate buffer, pH 7.5, containing photoaffinity analogues of NAD^+ at different concentrations. Before and after photoirradiation, 2-μL aliquots of the irradiation mixture were withdrawn and the enzyme activity was assayed. The enzyme was stable when it was photolyzed under the same conditions in the absence of photoprobes.

Results and Discussion

Characterization of (A)-2-Azido-NAD^+ and (A)-8-Azido-NAD^+. The structures of (A)-2-azido-NAD^+ and (A)-8-azido-NAD^+ are shown in Figure 1. The absorption spectra of the two compounds in 10 mM phosphate buffer (pH 7.5) are shown in parts A and B of Figures 2, respectively. In the presence of ethanol (0.5%) and yeast alcohol dehydrogenase (5 μg/mL), these NAD^+ analogues are reduced immediately. The absorption spectrum of (A)-2-azido-NADH (Figure 2A) was characterized by an increase of absorbance in the 340-nm region and a red shift of the absorbance maximum from 268 to 272 nm as pointed out previously by Kim and Haley (1990).

[1] Abbreviations: (A)-2-azido-NAD^+, nicotinamide 2-azidoadenine dinucleotide; (A)-8-azido-NAD^+, nicotinamide 8-azidoadenine dinucleotide; (A)-arylazido-β-alanyl-NAD^+, A3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl] nicotinamide adenine dinucleotide; (N)-arylazido-β-alanyl-NAD^+, N3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl nicotinamide adenine dinucleotide; (N)-arylazido-β-alanyl-$NADP^+$, N3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl] nicotinamide adenine dinucleotide phosphate; (A)-(4-benzoylbenzoyl)-NAD^+, A3′-*O*-[(4-benzoylbenzoyl] nicotinamide adenine dinucleotide phosphate; (N)-3-azido-PyAD^+, 3-azidopyridine adenine dinucleotide; 5′-FSBA, 5′-[(fluorosulfonyl)benzoyl]adenosine; NMN, nicotinamide mononucleotide.



(A)-2-azido NAD$^+$ X = N_3 , Y = H

(A)-8-azido NAD$^+$ X = H, Y = N_3

FIGURE 1: The structures of (A)-2-azido-NAD$^+$ and (A)-8-azido-NAD$^+$.

(A)-8-Azido-NADH has an absorption peak at 340 nm and also a red shift of the absorbance maximum from 274 to 280 nm (Figure 2B).

Figure 2C shows the spectral changes of (A)-2-azido-NAD$^+$ upon irradiation using a tungsten projector lamp (DVY 120 V, 650 W). The spectral changes were similar to those reported by Kim and Haley (1990) except that the isosbestic points were at 242, 262, and 329 nm, slightly different from those obtained upon exposure to a Mineralight UVSL-25 (254-nm) light. The spectral changes of (A)-8-azido-NAD$^+$ upon irradiation using a tungsten lamp are shown in Figure 2D. These photodependent spectral changes indicate a conversion of the azido group of the nucleotide analogue to a nitrene species upon light irradiation.

As indicated above, the compounds used in this study have spectral properties very similar to those reported by other groups. The identity of compounds synthesized in our laboratory as (A)-2-azido-NAD$^+$ and (A)-8-azido-NAD$^+$ was further established on the basis of several lines of evidences. First, these compounds were synthesized by the coupling of 2-azido-AMP or 8-azido-AMP to NMN. Therefore, they should be (A)-2-azido-NAD$^+$ and (A)-8-azido-NAD$^+$. The 8-azido-AMP was purchased from Sigma Chemical Co. The 2-azido-AMP was synthesized in our laboratory, and its identity was confirmed on the basis of known chemical data for this compound. Second, mass spectral analysis shows that our compounds have molecular weights identical with those for the expected structures. Third, these compounds were shown to be azido derivatives because they are photolabile as described above, and also these compounds, especially (A)-2-azido-NAD$^+$, undergo azido–tetrazole transitions by spectral analysis. Figure 3A,B shows the UV/vis absorption spectra of (A)-2-azido-NAD$^+$ obtained when the compound was kept at 4 °C in 0.1 N HCl or 0.1 N NaOH for 18 h and then neutralized. The spectral changes were very similar to those reported for 2-azido-AMP (Macfarlane et al., 1982) in that after neutralization a slow transition occurred to produce an intermediate spectrum. As indicated by Macfarlane et al. (1982), the form favored by acid is the azido derivative and is photolabile and the form favored by alkali is the tetrazole derivative and is not photolabile. These characteristic absorption spectral changes indicate again that this compound is indeed (A)-2-azido-NAD$^+$. We did not detect significant spectral transition for (A)-8-azido-NAD$^+$ after neutralization. However, we did observe a red shift of the absorbance maximum from 274 to 280 nm for neutralized solution containing (A)-8-azido-NAD$^+$ preincubated in 0.1 N NaOH (Figure 3C). The nature of this spectral shift is not currently understood. It is thought that alkali treatment may change the structure of the 8-azido-adenine ring significantly because the resulting compound(s) is no longer a substrate of yeast alcohol dehydrogenase (see below). Fourth, these two compounds synthesized in our laboratory are NAD$^+$ analogues as they are shown to be substrates of yeast alcohol dehydrogenase (as described above) and substrates of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (see below). Interestingly, both the azido and the tetrazole forms of (A)-2-azido-NAD$^+$ can be reduced by yeast alcohol dehydrogenase (results not shown). However, after incubation in 0.1 N NaOH, (A)-8-azido-NAD$^+$ is converted to a compound that has a different UV absorption maximum (Figure 3C) and cannot be reduced by yeast alcohol dehydrogenase.

Similar photodependent spectral changes were found for (A)-2-azido-NADH or (A)-8-azido-NADH when compared to (A)-2-azido-NAD$^+$ or (A)-8-azido-NAD$^+$ (results not shown). These results indicate that the azido group remains unchanged upon the reduction of NAD$^+$ analogues to NADH analogues by yeast alcohol dehydrogenase.

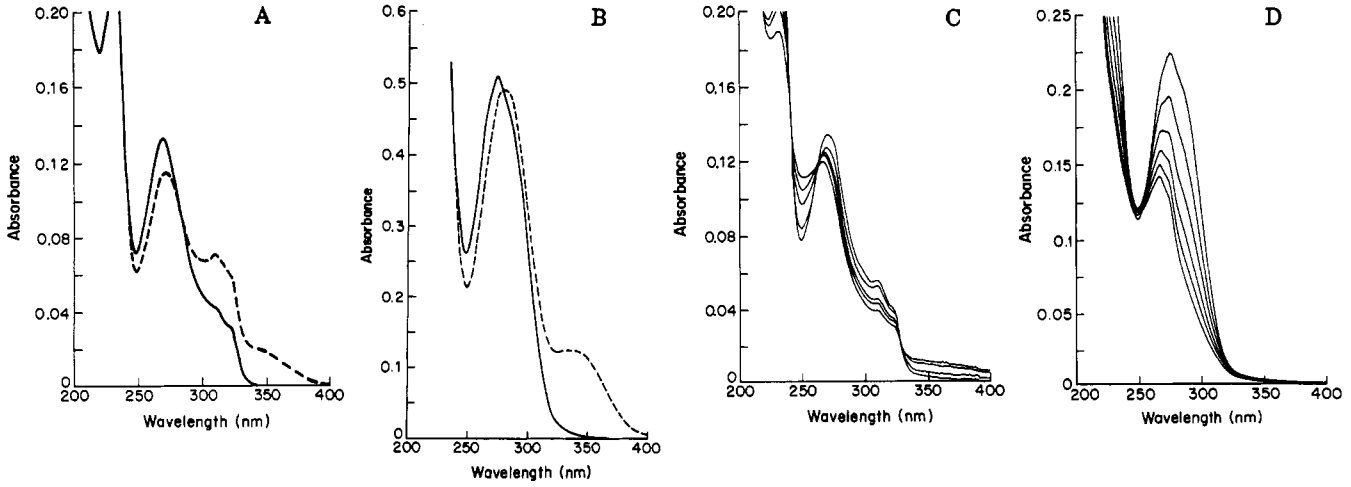


FIGURE 2: Spectral properties of (A)-2-azido-NAD(H) and (A)-8-azido-NAD(H). (A) Absorption spectra of (A)-2-azido-NAD$^+$ (—) and (A)-2-azido-NADH (---). (B) Absorption spectra of (A)-8-azido-NAD$^+$ (—) and (A)-8-azido-NADH (---). (C) Absorption spectral changes of (A)-2-azido-NAD$^+$ upon irradiation with a tungsten projector lamp (DVY 120 V, 650 W) at 1-min intervals. (D) Absorption spectral changes of (A)-8-azido-NAD$^+$ upon irradiation with a tungsten projector lamp (DVY 120 V, 650 W) at 1-min intervals.

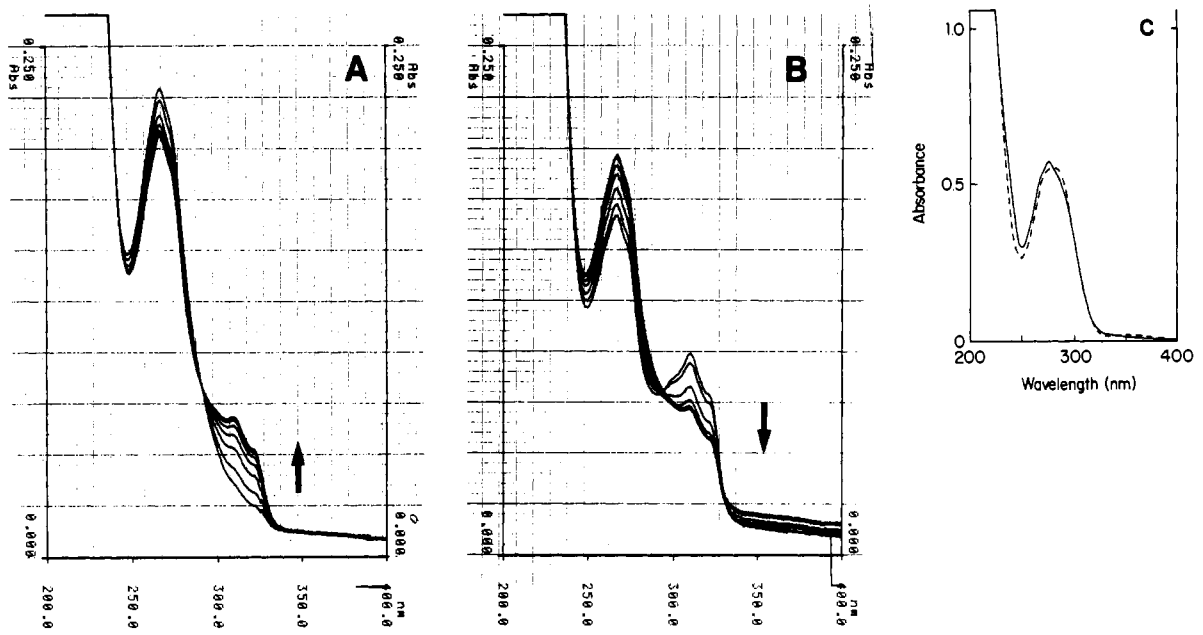


FIGURE 3: Azido–tetrazole transitions of azido-NAD$^+$ analogues. (A) Spectral changes of (A)-2-azido-NAD$^+$ after neutralization of 50 μL of analogue (preincubated at 0.1 N HCl at 4 °C for 18 h) by dilution with 0.95 mL of 50 mM phosphate buffer, pH 7.5. Spectra were taken at 0, 1, 5, 14, 24, 34, 47, and 54 min after neutralization. The arrow is used to indicate the sequence of spectral scanning. (B) Spectral changes of (A)-2-azido-NAD$^+$ after neutralization of 50 μL of analogue (preincubated at 0.1 N NaOH at 4 °C for 18 h) by dilution with 0.95 mL of 50 mM phosphate buffer, pH 7.5. Spectra were taken at 0, 3, 10, 20, 30, and 50 min after neutralization. (C) Spectra of (A)-8-azido-NAD$^+$ after neutralization of 50 μL of the compound [either preincubated at 0.1 N HCl (—) or 0.1 N NaOH (- - -) at 4 °C for 18 h] by dilution with 0.95 mL of 50 mM phosphate buffer, pH 7.5. There was no time-dependent spectral change for (A)-8-azido-NAD$^+$.

Table I: (A)-2-Azido-NADH and (A)-8-Azido-NADH as Substrates of Rat Liver NAD(P)H:Quinone Acceptor Oxidoreductase[a]

electron donor	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}·μM^{-1})
NADH	6.2	11 520	1858
(A)-2-azido-NADH	14.3	10 020	701
(A)-8-azido-NADH	18.5	5460	295

[a] The kinetic parameters were determined at 25 °C in a mixture containing 50 mM sodium phosphate, pH 7.4, 10 μM menadione, 77 μM cytochrome *c*, and variable concentrations of NADH or its analogues.

(A)-2-Azido-NADH and (A)-8-Azido-NADH as Substrates of NAD(P)H:Quinone Acceptor Oxidoreductase. As described above, (A)-2-azido-NAD$^+$ and (A)-8-azido-NAD$^+$ are very good substrates of yeast alcohol dehydrogenase. Since the activity of the quinone reductase was determined by measuring the reduction of cytochrome *c*, by including ethanol and yeast alcohol dehydrogenase in the assay mixture, the formed (A)-2-azido-NADH and (A)-8-azido-NADH were shown to be electron donors of this reductase (Table I). Under our assay conditions, the k_{cat} value for (A)-2-azido-NADH is very similar to that for NADH and the K_m value for (A)-2-azido-NADH is 2.3 times that for NADH. (A)-8-Azido-NADH is also a substrate for the rat liver NAD(P)H:quinone acceptor oxidoreductase with the k_{cat} value approximately $^1/_2$ and K_m value 3 times those for NADH, respectively. These results demonstrate that these two NADH analogues can bind to the nicotinamide nucleotide binding site of the enzyme by acting as the electron donors.

Photodependent Inhibition of NAD(P)H:Quinone Acceptor Oxidoreductase by (A)-2-Azido-NAD$^+$ and (A)-8-Azido-NAD$^+$. It is recognized that these compounds, especially (A)-2-azido-NAD$^+$, undergo an azide–tetrazole transition upon increasing pH, and they may be present as a mixture of azido and tetrazole derivatives at neutral pH (as discussed above). Although the form favored by acid is the azide form, which is photoreactive, an azide–tetrazole equilibrium will be reached in a time-dependent fashion upon introduction of the azide form to the 50 mM phosphate buffer, pH 7.5, in which the enzyme is kept and in which the enzyme assay is performed. In order to generate consistent results, we dissolved NAD$^+$ analogues in water and used them without other manipulation. Clear photodependent inhibition of rat liver NAD(P)H:quinone acceptor oxidoreductase by (A)-2-azido-NAD$^+$ and (A)-8-azido-NAD$^+$ was observed under this experimental condition (Figure 4). (A)-2-Azido-NAD$^+$ is a much more potent inhibitor than (A)-8-azido-NAD$^+$. A 50% inhibition of the activity can be obtained through a 2-min irradiation in the presence of 12 μM (A)-2-azido-NAD$^+$, while only a 42% inhibition is obtained in the presence of 2 mM (A)-8-azido-NAD$^+$. The photodependent inhibition of the enzyme by these compounds can be prevented by the presence of NADH during the photoirradiation process (Table II), suggesting that these compounds do bind to the active site of the enzyme upon photoirradiation. It is not unexpected that (A)-8-azido-NAD$^+$ is not as good an inhibitor as (A)-2-azido-NAD$^+$. The presence of the azido group at the C-8 position of the adenine ring places the adenine in an syn conformation, which is not a favorable conformation for binding to the nicotinamide nucleotide binding site. This result agrees with those from kinetic studies showing that (A)-8-azido-NADH is a less effective electron donor of the quinone reductase than (A)-2-azido-NADH (Table I). A 22% inhibition was observed when the enzyme was assayed in the presence of 18.8 μM prephotolyzed (A)-2-azido-NAD$^+$. This inhibition of the enzyme is probably resulted from the photolyzed derivatives because a 94% inhibition of the enzyme when it was photolyzed in the presence of 16 μM (A)-2-azido-NAD$^+$ (Table II). Inhibition was not observed when the enzyme was assayed in the presence of 1.5 mM of prephotolyzed (A)-8-azido-NAD$^+$ (Table II). These results and those obtained from the protection experiments indicate that the photodependent inhibition caused by (A)-2-azido-NAD$^+$ or (A)-8-azido-NAD$^+$ resulted

Table II: Effects of Substrates on Photoinactivation of NAD(P)H:Quinone Acceptor Oxidoreductase by Photoaffinity Analogues of NAD^+

photoprobes[a] (μM)	additions (μM)	remaining act. (%)
		100
(A)-2-azido-NAD^+ (16)		6
(A)-2-azido-NAD^+ (16)	NADH (200)	91
	menadione (200)	55
(A)-2-azido-NAD^+ (16)	menadione (200)	41
(A)-8-azido-NAD^+ (773)		70
(A)-8-azido-NAD^+ (1546)		58
(A)-8-azido-NAD^+ (1546)	NADH (200)	95
(A)-8-azido-NAD^+ (1546)	menadione (200)	20
(A)-8-azido-NAD^+ (773)	menadione (200)	26
(A)-8-azido-NAD^+ (773)	menadione (120)	36
(A)-8-azido-NAD^+ (773)	menadione (40)	32
(A)-8-azido-NAD^+ (773)	menadione (40)	57
	NADH (200)	
	menadione (40)	65
(A)-2-azido-NAD^+ (18.8) (prephotolyzed)[b]		78
(A)-8-azido-NAD^+ (1500) (prephotolyzed)		110

[a] The irradiation time was 2 min. [b] A solution containing NAD^+ analogue at the indicated concentration in 50 mM phosphate buffer, pH 7.5, was irradiated for 2 min. The quinone reductase was then introduced and assayed according to method described in the Materials and Methods section.

from incorporation of these compounds at the active site of this quinone reductase upon photoirradiation.

It was also found that the photodependent inhibition of this quinone reductase by (A)-8-azido-NAD^+ was enhanced in the presence of the substrate, menadione, during irradiation (see Table II and compare Figure 4B,C). As shown in Table II and Figure 4C, the activity of the quinone reductase decreased even when it was photolyzed with menadione alone. The nature of this photodependent inhibition of the quinone reductase by menadione is not presently understood. One possibility is that the photolysis of menadione results in the formation of dimers of menadione as shown by Werbin and Strom (1968) and that these dimers have structures like dicoumarol and could inhibit this quinone reductase in a fashion similar to dicoumarol. Dicoumarol has been shown to be a potent competitive inhibitor of this enzyme with respect to NAD(P)H oxidation (Lind et al., 1979). Menadione has also been shown to be able to sensitize the photooxidation of protein (Krishna et al., 1987). The quinone binding site of NAD(P)H:quinone acceptor oxidoreductase could also be destroyed through such a photooxidation process. In contrast to its effect on the photodependent inhibition of this quinone reductase by (A)-8-azido-NAD^+, menadione protected the enzyme from inhibition by (A)-2-azido-NAD^+ (Table II). The activity of the enzyme when photolyzed with both (A)-2-azido-NAD^+ and menadione was similar to that of the enzyme photolyzed with menadione alone. The photodependent inhibition of the quinone reductase by (A)-8-azido-NAD^+ in the presence of menadione can be prevented by the presence of NADH during irradiation (Table II), indicating that (A)-8-azido-NAD^+ binds to the nicotinamide nucleotide binding site of the quinone reductase even in the presence of menadione.

Although Hall et al. (1972) have reported that NAD(P)H:quinone acceptor oxidoreductase reacts with its electron donor and acceptor mainly according to a "ping-pong" mechanism, Hosoda et al. (1974) have revealed that the quinone reductase can form an enzyme–menadione complex when menadione is present and the FAD prosthetic group is rapidly reduced by NAD(P)H at a rate almost comparable with that of the enzyme in the absence of menadione. Hosoda

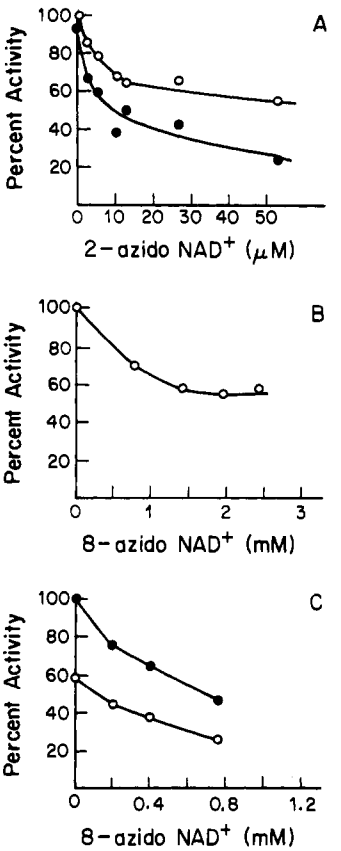


FIGURE 4: Photodependent inhibition of NAD(P)H:quinone acceptor oxidoreductase by (A)-2-azido-NAD^+ and (A)-8-azido-NAD^+. (A) The enzyme (23 μg) was incubated with (A)-2-azido-NAD^+ at the indicated concentrations in 150 μL of 50 mM sodium phosphate buffer, pH 7.5, and irradiated for 1 (○) or 2 min (●). The enzyme activity before irradiation was taken as 100%. (B) The enzyme (15 μg) was incubated with (A)-8-azido-NAD^+ at the indicated concentrations in 100 μL of 50 mM sodium phosphate buffer, pH 7.5, and irradiated for 2 min. (C) The enzyme was incubated with (A)-8-azido-NAD^+ at the indicated concentrations and menadione at 0.2 mM and irradiated for 2 min. (○) The percent activity was calculated by taking the enzyme activity of the sample without menadione before irradiation as 100%; (●) the percent activity was calculated by taking the enzyme activity of the sample containing 0.2 mM menadione after irradiation as 100%. This latter calculation allows us to examine the effect of (A)-8-azido-NAD^+ on this enzyme in the presence of 0.2 mM menadione.

et al. (1974) have also found that the formed $FADH_2$ is oxidized by the bound menadione in a much more sluggish manner. These findings indicate that, first, NAD(P)H:quinone acceptor oxidoreductase can catalyze a reaction following a ternary complex mechanism, second, the menadione binding site in the enzyme is different from the NAD(P)H binding site, and third, the reduction of quinones or the dissociation of the hydroquinones (or quinones) from the enzyme is the rate-limiting step of the reaction. This last point may explain why the enzyme activity is lower when the enzyme is preincubated with menadione. Furthermore, menadione may enhance the binding of (A)-8-azido-NAD^+ to the quinone reductase by the formation of a stable enzyme–menadione–(A)-8-azido-NAD^+ ternary complex, resulting in a stronger inhibition of the enzyme than the sample photolyzed in the absence of menadione. Since no additional inhibition was found when the enzyme was photolyzed with (A)-2-azido-NAD^+ and menadione than when the enzyme was photolyzed with menadione alone, it is though that menadione prevents (A)-2-azido-NAD^+ from labeling the enzyme. These results indicate that these two NAD^+ analogues bind to the quinone

reductase differently and the position of the azido group on the adenine ring of the NAD^+ molecule has a definite influence on the binding of these NAD^+ analogues to this enzyme. Identification of the sites modified by (A)-2-azido-NAD^+ and (A)-8-azido-NAD^+ may provide useful information concerning the mechanism of the interaction of menadione with the nucleotide coenzymes. Our affinity-labeling studies of the quinone reductase using 5'-FSBA revealed that the binding affinity of this probe was also enhanced in the presence of menadione (unpublished results).

Interaction of the Rat Liver NAD(P)H:Quinone Acceptor Oxidoreductase with (A)-Arylazido-β-alanyl-NAD^+, (N)-Arylazido-β-alanyl-NAD^+, (A)-(4-Benzoylbenzoyl)-NAD^+, and (N)-3-Azido-NAD^+. These four compounds, at up to 3 mM, did not inhibit the quinone reductase either in the presence or in absence of menadione upon light irradiation. The synthesis of (A)-arylazido-β-alanyl-NAD^+ has been previously reported (Chen & Guillory, 1977). (N)-arylazido-β-alanyl-NAD^+ was prepared by removing the 2'-phosphate group from (N)-arylazido-β-alanyl-$NADP^+$ by alkaline phosphatase treatment (Deng et al., 1990a). The synthesis of (N)-arylazido-β-alanyl-$NADP^+$ is as previously published (Chen & Guillory, 1980). (N)-3-Azido-$PyAD^+$ was prepared by exchanging the nicotinamide ring of NAD^+ with 3-azidopyridine through catalysis by NADase as described by Hixson and Hixson (1973). (A)-(4-Benzoylbenzoyl)-NAD^+ was prepared by coupling 4-benzoylbenzoic acid to NAD^+ through carbodiimidazole catalysis (unpublished results).

Interestingly, (A)-arylazido-β-alanyl-NADH and (N)-arylazido-β-alanyl-NADH, obtained by the reduction of the NAD^+ analogues by rabbit muscle glyceraldehyde-3-phosphate dehydrogenase in the presence of glyceraldehyde 3-phosphate [for method see Chen et al. (1984)], are substrates of the rat liver NAD(P)H:quinone acceptor oxidoreductase. The k_{cat}/K_m ratios are 1037 $min^{-1}\cdot\mu M^{-1}$ and 1643 $min^{-1}\cdot\mu M^{-1}$ for (A)-arylazido-β-alanyl-NADH and (N)-arylazido-β-alanyl-NADH, respectively, suggesting that they are more effective electron donors of the quinone reductase than (A)-2-azido-NADH and (A)-8-azido-NADH (see Table I). (A)-(4-Benzoylbenzoyl)-NAD^+ is a poor substrate of yeast alcohol dehydrogenase or rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. We could not produce enough (A)-(4-benzoylbenzoyl)-NADH to test whether it is a substrate of NAD(P)H:quinone acceptor oxidoreductase. (N)-3-Azido-$PyAD^+$ is not a substrate of yeast alcohol dehydrogenase or rabbit muscle glyceraldehyde-3-phosphate dehydrogenase.

Out of seven NAD^+ analogues, three compounds were found to be useful for labeling the nicotinamide nucleotide binding site of the rat liver NAD(P)H:quinone acceptor oxidoreductase. They are 5'-FSBA, (A)-2-azido-NAD^+, and (A)-8-azido-NAD^+. On the basis of the available results, we propose that in the nicotinamide nucleotide binding site of this quinone reductase the regions interacting with the ribose moieties of the nicotinamide nucleotides are less constrained. The ineffectiveness of (A)-arylazido-β-alanyl-NADH and (N)-arylazido-β-alanyl-NADH as photoaffinity probes to label the nicotinamide nucleotide binding site of the quinone reductase may be due to the fact that the arylazido-β-alanyl group of the analogues is not directly in contact with the protein. Such a model would explain why the nicotinamide nucleotide binding site of the enzyme can accommodate NADH as well as NADPH.

At the present time, we have seven different NAD^+ affinity and photoaffinity analogues that have the reactive group at different parts of the NAD^+ molecules. We anticipate that studies using these compounds would provide structural information concerning different regions of the nicotinamide nucleotide binding sites of different NAD(H)-dependent enzymes. We have actually found that different enzymes react distinctly with different compounds. In a separate study (Chen & Guillory, 1981; Deng et al., 1990a,b), only two of seven NAD^+ analogues, (A)-arylazido-β-alanyl-NAD^+ and (N)-arylazido-β-alanyl NAD^+, were shown to be useful compounds to investigate the active site of the mitochondrial NADH dehydrogenase. As another example, (A)-8-azido-NAD^+, (A)-2-azido-NAD^+, (A)-arylazido-β-alanyl-NAD^+ (Chen & Guillory, 1977), and (A)-(4-benzoylbenzoyl)-NAD^+ (unpublished observation) were shown to be substrates of yeast alcohol dehydrogenase. However, we have found that (N)-arylazido-β-alanyl-NAD^+ is not a substrate of yeast alcohol dehydrogenase (Deng et al., 1990a). We have recently found that (A)-2-azido-NAD^+, (A)-8-azido-NAD^+, (A)-arylazido-β-alanyl-NAD^+, and (N)-arylazido-β-alanyl-NAD^+ are good substrates of the rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. These differences in the reactivities of seven NAD^+ probes with different nicotinamide nucleotide dependent enzymes suggest clear differences among the active sites of these enzymes.

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Neutral Imidazole Is the Electrophile in the Reaction Catalyzed by Triosephosphate Isomerase: Structural Origins and Catalytic Implications[†,‡]

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ABSTRACT: To illuminate the role of histidine-95 in the catalytic reaction mediated by triosephosphate isomerase, ^{13}C and ^{15}N NMR titration studies have been carried out both on the wild-type enzyme and on a mutant isomerase in which the single remaining histidine (that at the active site) has been isotopically enriched in the imidazole ring. ^{15}N NMR has proved especially useful in the unambiguous demonstration that the imidazole ring of histidine-95 is uncharged over the entire pH range of isomerase activity, between pH 5 and pH 9.9. The results require that the first pK_a of histidine-95 is below 4.5. This abnormally low pK_a rules out the traditional view that the positively charged imidazolium cation of histidine-95 donates a proton to the developing charge on the substrate's carbonyl oxygen. ^{15}N NMR experiments on the enzyme in the presence of the reaction intermediate analogue phosphoglycolohydroxamate show the presence of a strong hydrogen bond between $N^{\epsilon 2}$ of histidine-95 and the bound inhibitor. These findings indicate that, in the catalyzed reaction, proton abstraction from C-1 of dihydroxyacetone phosphate first yields an enediol*ate* intermediate that is strongly hydrogen bonded to the neutral imidazole side chain of histidine-95. The imidazole proton involved in this hydrogen bond then protonates the enediolate, with the transient formation of the enediol-imidazol*ate* ion pair. Abstraction of the hydroxyl proton on O-1 now produces the other enediolate intermediate, which collapses to give the product glyceraldehyde 3-phosphate. This initially surprising sequence is more reasonable when it is recognized that the pK_a values of the enediol and the perturbed pK_a[2] of the imidazole ring of histidine-95 may be rather close to each other, allowing for two facile and rapid proton transfers that interconvert the two enediolates. To our knowledge, this is the first reported example of the participation of an imidazolate side chain in an enzyme-catalyzed reaction. The imidazole ring of histidine-95 lies at the amino terminus of a short α-helix that will, in accord with what is known from the behavior of substituted imidazoles in solution, lower both the first and the second pK_a values of the side chain of histidine-95.

Triosephosphate isomerase is a glycolytic enzyme that catalyzes the interconversion of (*R*)-glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). As shown in Figure 1, glutamate-165 is the catalytic base responsible for proton abstraction from carbon (Waley et al., 1970; Hartman, 1971; de la Mare et al., 1972; Banner et al., 1975; Lolis et al., 1990), while histidine-95 is believed to be the catalytic acid (Belasco & Knowles, 1980; Nickbarg et al., 1988; Komives et al., 1991). The results of Rieder and Rose (1959) and of Bloom and Topper (1956) are consistent with the intermediacy of an enediol(ate) in the catalyzed reaction (Figure 1).

The role of histidine-95 in the catalytic mechanism of triosephosphate isomerase has been the subject of considerable study and speculation (Belasco & Knowles, 1980; Komives et al., 1991; Nickbarg et al., 1988). There are at least three functions that could be performed by this residue. First, histidine-95 may polarize the carbonyl group in the substrate DHAP, facilitating the abstraction of the *pro-R* hydrogen at C-1 by glutamate-165. Earlier studies by Belasco and Knowles (1980) demonstrated that the carbonyl group of DHAP is indeed polarized on binding to the enzyme, and Komives et al. (1991) have recently shown that histidine-95 is responsible for this polarization in the wild-type enzyme. The substrate is oriented in the active site so that the *pro-R* hydrogen is perpendicular to the plane defined by O-1, C-1, C-2, and O-2,

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