Photoaffinity Labeling of Lactate Dehydrogenase by the Carbene Derived from the 3-Diazirino Analogue of Nicotinamide Adenine Dinucleotide†

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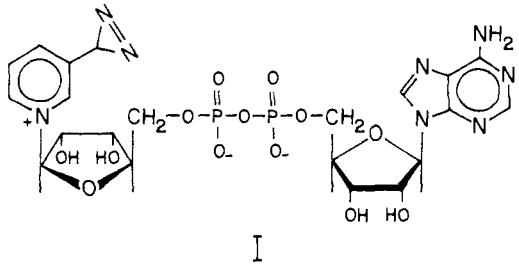
ABSTRACT: The 3-diazirino analogue of NAD^+, DAD^+, in which the diazirine group replaces the carboxamide in an almost isosteric substitution, has been synthesized as a photoaffinity reagent for dehydrogenases. With lactate dehydrogenase, the K_{diss} is 4 mM (compare the NAD^+ K_{diss} of 0.6 mM). On photolysis, three types of interaction can be discerned and separately quantitated by using [^{3}H]DAD^+: noncovalent binding of the photoproducts of DAD^+, which is removed by protein denaturation; nonspecific covalent labeling, which is eliminated by the presence of a scavenger such as glutathione; specific covalent labeling at the active site, which is prevented by competition from the natural ligands. The photolabeling efficiency (sites covalently labeled/sites initially occupied) is ~0.3. It is evident that the carbene generated from DAD^+ is more effective than that from the more bulky 3-diazoacetate or than the less reactive nitrene derived from the 3-azido-NAD^+ analogue.

The first experiments that used photogenerated reagents for the labeling of the ligand binding sites of macromolecules, a concept introduced by Westheimer and co-workers, involved α-diazocarbonyl compounds as photoprecursors of carbenes (Singh et al., 1962; Shafer et al., 1966). While these early studies demonstrated the attractiveness and feasibility of the approach, the chemical instability of the diazoacyl precursors and the facile Wolff rearrangement of the generated α-ketocarbenes limited the effectiveness of the method in the sense that relatively low yields of product derived directly from the photogenerated carbene were obtained. In an effort to circumvent these problems, aryl azides were introduced (Fleet et al., 1969) on the basis of the thermal stability of the precursor and the small tendency of aryl nitrenes to rearrange to less reactive species. It has become increasingly clear, however, that the lesser reactivity of nitrenes (compared with the corresponding carbenes) can cause problems in cases where the photolabile ligand is not bound extremely tightly to its receptor site (Bayley & Knowles, 1978a,b), and a second generation of carbene precursors has therefore been developed. Thus, it has been suggested that 3-aryl-3*H*-diazirines (Smith & Knowles, 1973, 1975), 2-diazo-3,3,3-trifluoropropionates (Chowdhry et al., 1976), α-diazophosphonates (Goldstein et al., 1976), and tosyldiazoacetates (Chowdhry & Westheimer, 1978) have properties that recommend their use as efficient carbene precursors for photoaffinity labeling studies. We report here on the use of one of these, the diazirine, which has been incorporated into NAD^+ [1] in an almost isosteric replacement of the 3-carboxamido group of the nicotinamide ring. The effectiveness of the resulting photolabile NAD^+ analogue (I) in labeling lactate dehydrogenase is described.



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Materials and Methods

NAD^+, NADH, lithium lactate (both L and D enantiomers), reduced glutathione, sodium thioglycolate, pig brain NAD^+ glycohydrolase (NADase), and L-lactate dehydrogenase (from rabbit muscle, lyophilized and buffer free, Type XI) were obtained from Sigma Chemical Co. Thiolactic acid and 3-pyridinecarboxaldehyde were from Aldrich. Hydroxylamine-*O*-sulfonic acid was prepared by the method of Matsuguma & Audrieth (1957) and *tert*-butyl hypochlorite was synthesized according to Mintz & Walling (1973). All other reagents and buffer components were commercial grades of the highest purity. [2,8-^{3}H]NAD^+ (sp radioact 0.32 Ci $mmol^{-1}$) was obtained from New England Nuclear.

3-Pyridyl-3*H*-diazirine was synthesized essentially as described in method 4 of Smith & Knowles (1975). Chromatography of the product on silica gel eluted with CCl_4–Et_2O (70:30 v/v) and evaporation of the eluant under reduced pressure gave 3-pyridyl-3*H*-diazirine as a yellow oil which was stored in the dark in *n*-pentane at −20 °C until required. ^{1}H NMR (in $CDCl_3$) δ 8.58 (dd, 1 H, J = 4.8 and 1.8 Hz), 8.42 (dd, 1 H, J = 2.0 and 0.7 Hz), 7.31 (ddd, 1 H, J = 7.9, 5.0, and 0.7 Hz), 7.13 (dt, 1 H, J = 7.9 and 2.0 Hz), 2.12 (s, 1 H). The infrared spectrum (liquid film) included a strong band at 1590 cm^{-1} (N=N). The ultraviolet spectrum (hexane) showed a strong maximum at 265 nm and smaller peaks at 358, 368, and 378 nm.

3-(3*H*-Diazirino)pyridine adenine dinucleotide (DAD^+) was prepared by the exchange reaction catalyzed by NADase. This reaction can conveniently be monitored by TLC on fluorescent poly(ethylenimine)–cellulose plates (Brinkman) developed with isobutyric acid–concentrated ammonia–water (66:1:33 v/v) or by high-pressure liquid chromatography on AX-corasil anion-exchange columns (Waters Associates) eluted with 15 mM sodium phosphate buffer, pH 7.

3-Pyridyl-3*H*-diazirine (41 mg, 344 μmol) was dissolved in distilled water (4 mL) containing NAD^+ (40 mg, 55 μmol), and the exchange reaction was initiated by the addition of solid NADase (50 mg, 0.75 unit). The mixture was stirred in the dark at room temperature for 14 h. At this point, the exchange reaction is ~60% complete and very little hydrolysis has occurred. Addition of a further portion of NADase (15 mg,

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[1] Abbreviations used: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; NADase, NAD^+ glycohydrolase from pig brain; DAD^+, 3-(3*H*-diazirino)pyridine adenine dinucleotide.

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Table I: Attachment of [^{3}H]DAD$^+$ to Lactate Dehydrogenase after Irradiation[a]

	conditions	cpm/A_{280} in protein fractions	[(mol of DAD$^+$ attached)/(mol of active sites)] × 100
enzyme plus DAD$^+$	*b*	920 ± 40[c]	16
enzyme plus DAD$^+$	*h*	614 ± 16	11
enzyme plus DAD$^+$	*b, d*	48 ± 2	0.8
enzyme plus DAD$^+$ plus NAD$^+$	*b, e*	497 ± 26	8.8
enzyme plus DAD$^+$ plus NAD$^+$	*b, f*	490 ± 11	8.7
enzyme plus DAD$^+$ plus NAD$^+$ plus oxalate	*b, g*	165 ± 5	2.9
enzyme plus DAD$^+$ plus NAD$^+$ plus oxalate	*b, g, l*	78 ± 2	1.4
enzyme plus DAD$^+$ plus NAD$^+$ plus oxalate	*h*	50 ± 20	0.9
enzyme plus DAD$^+$	*b, i*	353 ± 8	6.2
enzyme plus DAD$^+$	*b, j*	363 ± 21	6.4
enzyme plus DAD$^+$	*b, k*	727 ± 130	12
enzyme plus DAD$^+$	*b, l*	753 ± 55	13
enzyme plus DAD$^+$ plus NAD$^+$	*b, l, f*	349 ± 23	6.2
enzyme plus DAD$^+$ plus L-lactate	*b, m*	1267 ± 8	22
enzyme plus DAD$^+$ plus oxalate	*b, n*	900 ± 41	16
enzyme plus DAD$^+$ plus L-lactate	*h, m*	1018 ± 3	18
enzyme plus DAD$^+$ plus D-lactate	*h, m*	620 ± 5	11

[a] Enzyme concentration, 1.1 mM (in subunits); DAD$^+$ concentration, 1.2 mM; 100 mM Tris-HCl buffer, pH 8.0; 4 °C; 20–90-min irradiation at 350 nm. [b] After gel filtration on Sephadex G-25, in 100 mM Tris-HCl buffer, pH 8.0. [c] Mean of four determinations. [d] No photolysis. [e] NAD$^+$ concentration, 4 mM. [f] NAD$^+$ concentration, 50 mM. [g] NAD$^+$ concentration, 38 mM; oxalate concentration, 22 mM. [h] After gel filtration as in footnote *b*, in the presence of 0.1% (w/v) sodium dodecyl sulfate. [i] DAD$^+$ prephotolyzed for 6 min before addition of the enzyme and further incubation in the dark for 1 h at 37 °C. [j] DAD$^+$ prephotolyzed for 100 min before addition of the enzyme and then photolysis as in footnote *a*. [k] Reduced glutathione (5 mM) present as a scavenger. [l] Reduced glutathione (25 mM) present as a scavenger. [m] Lactate concentration, 50 mM. [n] Oxalate concentration, 50 mM.

0.225 unit) and stirring for another 8 h increase the conversion of NAD$^+$ into DAD$^+$ to greater than 80%. At the end of the incubation, the NADase was removed by centrifugation (12000*g*; 10 min), and the supernatant was washed once with chloroform (6 mL) and then chromatographed on a column (30 cm × 0.6 cm^2) of DEAE-cellulose equilibrated with 5 mM NH_4HCO_3, pH 8.4. Elution with a linear gradient (5–200 mM, 50 + 50 mL) of NH_4HCO_3 provided a mixture of DAD$^+$ and NAD$^+$ well separated from pyridine derivatives and adenosine diphosphoribose. Fractions containing the pyridine nucleotides were pooled, the solvent was evaporated, and the residue was dissolved in distilled water (2 mL). TLC demonstrated that DAD$^+$ was already ~90% pure at this stage, the other 10% being NAD$^+$. The NAD$^+$ was removed by incubating the solution (2 mL) for 2 h at room temperature with ethanol (0.5 mL) and yeast alcohol dehydrogenase (~1 mg). Rechromatography on DEAE-cellulose, as described above, separated DAD$^+$ from the NADH produced and gave a 60% overall yield of DAD$^+$ (based on NAD$^+$). This material was stored as a solution (16 mM) in distilled water at 4 °C in the dark. The DAD$^+$ migrated as a single spot in three different TLC systems that separate NAD$^+$ and DAD$^+$ [silica gel plates eluted with methanol–water (85:15 v/v); silica gel plates eluted with 2-propanol–concentrated ammonia–water (9:1:2 v/v); poly(ethylenimine) plates eluted with isobutyl alcohol–concentrated ammonia–water (66:1:33 v/v)]. DAD$^+$ was characterized by ^{1}H, ^{13}C, and ^{31}P NMR, the spectra being completely in accord with an intact adenine–ribose–pyrophosphate–ribose linkage to the 3-pyridyl-3*H*-diazirine moiety. ^{1}H NMR spectrum (in D_2O) δ 8.86 (d, 1 H, J = 6.5 Hz), 8.84 (d, 1 H, J = 1.3 Hz), 8.39 (s, 1 H), 8.1 (s, 1 H), 7.92 (dd, 1 H, J = 8.0 and 6.5 Hz), 7.70 (dt, 1 H, J = 8.1 and 1.4 Hz), 5.99 (m, 2 H), 4.5 (br m, 10 H), 2.60 (s, 1 H). The ultraviolet spectrum of DAD$^+$ has λ_{max} at 260 nm (ϵ = 17000) and 340 nm (ϵ = 623) with a pronounced shoulder between 290 and 320 nm. The $OD_{260/290}$ ratio for pure DAD$^+$ is 4.6, and this value can be used to monitor the progress of purification, especially from NAD$^+$.

The synthesis of the DAD$^+$ tritiated in the adenosine moiety was done as described above, using [2,8-^{3}H]NAD$^+$ (25 μCi) in the enzyme-catalyzed exchange reaction. The specific radioactivity of the [^{3}H]DAD$^+$ produced was 0.41 Ci/mol. Radiochemical purity was established by TLC on poly(ethylenimine)–cellulose as described above.

Binding Studies. The binding of [^{3}H]DAD$^+$ to rabbit muscle lactate dehydrogenase was investigated by microequilibrium dialysis as described by Englund et al. (1969). Experiments were performed at 4 °C in 67 mM Tris-HCl buffer, pH 8.0, and 0.2 M in NaCl. The distribution of ligand was determined by withdrawing duplicate samples (4–10 μL) from each compartment and counting them in a scintillation cocktail (10 mL) of toluene (850 mL), Liquifluor (50 mL), and Bio-Solv (100 mL; from Beckman). Further details are given in the legend to Figure 4.

Photolysis of DAD$^+$. A solution of DAD$^+$ (76 μM) in 50 mM sodium phosphate buffer, pH 7.0, was photolyzed in a 10-mm quartz optical cuvette at either 254 or 350 nm, 4 cm from the appropriate lamp of a Rayonet minireactor. Conversion of the diazirine to the linear diazo compound was monitored spectrophotometrically at 295 nm and reached a maximum after ~1 min (irradiation at 254 nm) or ~3 min (irradiation at 350 nm) [see Smith & Knowles (1975)].

Photolabeling of Lactate Dehydrogenase. Portions of buffered lactate dehydrogenase solution (200 μL) were mixed with [^{3}H]DAD$^+$ and other reagents as appropriate, to a final volume of 250 μL. These solutions were photolyzed at 4 °C in 1.0-mL Pyrex tubes (Corning), 2 cm from the 350-nm lamp in a Rayonet minireactor. Further details are given in the legend to Table I.

Analysis of Labeling. The photolyzed solutions (250 μL) and washings (100 μL) were loaded onto a column (8 mL) of Sephadex G-25 equilibrated with and eluted with 50 mM sodium phosphate buffer, pH 7.0. Fractions of 350–400 μL were collected. The distribution of radioactivity was determined by counting portions (25 μL) of each fraction in the Bio-Solv scintillation fluid (10 mL). Dehydrogenase concentrations were estimated from the absorbance at 280 nm [ϵ_{280} = 1.62 × 10^5 M^{-1} cm^{-1} (Fromm, 1963)]. For each fraction containing labeled protein, the absorbance and radioactivity measurements were repeated and provide the estimates of labeling given in Table I. For experiments in which the enzyme was denatured to remove noncovalently bound

material, sodium dodecyl sulfate was added to the photolyzed solution to a final concentration of 1% (w/v), and the column was eluted with 10 mM Tris-HCl buffer, pH 8.0, containing 0.1% (w/v) sodium dodecyl sulfate.

For the photolabeling time-course experiments, the enzyme was photolyzed as described above with [^{3}H]DAD$^+$. Withdrawn samples (25 μL) were incubated with NADH (100 μL, 11 mM) to remove noncovalently bound DAD$^+$ or its photoproducts prior to precipitation of the protein at 4 °C with 10% (w/v) trichloroacetic acid (125 μL). The precipitate was isolated by centrifugation, washed with water several times, and then dissolved in 100 mM Tris-HCl buffer (600 μL) containing sodium dodecyl sulfate (1% w/v), before counting in the Triton-based scintillation fluid (10 mL).

Photolabeling experiments conducted with a known amount of the enzyme–DAD$^+$ complex were performed by taking portions (15 μL) of solution directly from the equilibrium dialysis apparatus and photolyzing these in 1-mL Pyrex tubes as described above. After irradiation, the protein was precipitated with trichloroacetic acid, and the labeling was determined as described for the time-course experiment.

Assays. L-Lactate dehydrogenase was assayed as described by Kornberg (1955). L-Lactate was determined as described by Bergmeyer (1974).

Results and Discussion

Synthesis and Properties of DAD$^+$. 3-(3*H*-Diazirino)-pyridine adenine dinucleotide (DAD$^+$, I) was synthesized from 3-pyridyl-3*H*-diazirine and NAD$^+$ by the exchange reaction catalyzed by NADase as described by Biellmann et al. (1974). Although the (diazirino)pyridine was contaminated with 3-cyanopyridine, the latter is not a substrate for the exchange enzyme (Biellmann & Jung, 1970). The progress of the exchange reaction was followed by TLC and high-pressure liquid chromatography. The conversion of NAD$^+$ to DAD$^+$ was greater than 80%. After ion-exchange chromatography, remaining traces of NAD$^+$ were removed by treatment with alcohol dehydrogenase–ethanol, and the DAD$^+$ was repurified chromatographically. The final product showed undetectable amounts of NAD$^+$ in three TLC systems and was characterized by ultraviolet and NMR spectroscopy.

DAD$^+$ is inactive as a cofactor in the reactions catalyzed by alcohol dehydrogenase and by lactate dehydrogenase. The analogue is insensitive to borohydride, and high concentrations of dithionite reduce the diazirine rather than the pyridine ring. This low reactivity is expected for pyridinium compounds that lack an electron-withdrawing group in the 3 position (Anderson & Kaplan, 1959; Anderson et al., 1959).

Photolysis of DAD$^+$. As is expected for a 3-aryl-3*H*-diazirine (Smith & Knowles, 1975), DAD$^+$ is photolyzed rapidly by irradiation at either 350 or 254 nm. The changes in the ultraviolet absorption spectrum (notably at 295 nm) parallel those observed previously for other 3-aryl-3*H*-diazirines and show that fragmentation to the aryl carbene occurs concomitantly with photoisomerization to the linear diazo compound that is then itself photolyzed. Ultraviolet difference spectroscopy demonstrates that the diazo compound shows new absorbance bands at 303 and 372 nm. These bands are lost upon treatment with dilute acetic acid.

Binding of DAD$^+$ to Lactate Dehydrogenase. [^{3}H]DAD$^+$ binds to lactate dehydrogenase, giving a linear Scatchard plot that demonstrates noncooperative binding to the enzyme with a dissociation constant (pH 8.0; 4 °C) of 4.1 mM. This can be compared with NAD$^+$, which has a dissociation constant of 0.6 mM at pH 8.5 and 20 °C (Stinson & Holbrook, 1973). Some 77% of subunit sites [on the basis of A_{280} of the protein solution and the extinction coefficient of 1.62×10^5 M^{-1} cm^{-1} (Fromm, 1963)] were accessible to DAD$^+$. Equilibrium dialysis with NADH under the same conditions shows that ~78% of the subunit sites (estimated by measurement of A_{280} of the protein) are accessible to NADH. DAD$^+$ can therefore bind to all the sites that are available to NADH.

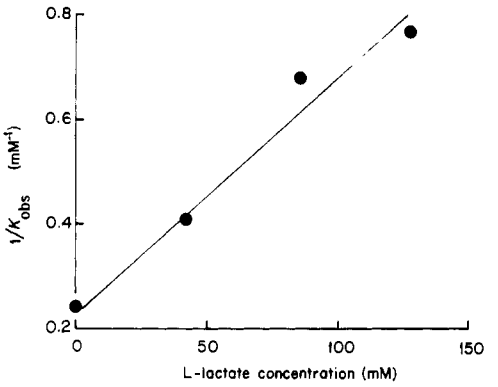

FIGURE 1: Plot of $1/K_{obsd}$ for the binding of DAD$^+$ to lactate dehydrogenase vs. the concentration of L-lactate.

In the presence of L-lactate, DAD$^+$ binds more tightly to the enzyme, as expected from the known ordered addition of ligands [NAD$^+$ and then lactate (Silverstein & Boyer, 1964; Zewe & Fromm, 1965)]. Assuming that the nucleotide binds before lactate in forming the nonproductive enzyme–DAD$^+$–lactate complex, the effect of added lactate upon the observed dissociation constant for DAD$^+$ is given by the equation $K_{obsd} = K_{enz\text{-}DAD^+}/(1 + [\text{lactate}]/K_{enz\text{-}DAD^+\text{-}lactate})$ (Holbrook, 1972). A plot of $1/K_{obsd}$ vs. [lactate] should therefore be a straight line. Such a plot is shown in Figure 1, and a dissociation constant of 49 mM can be obtained for the dissociation of L-lactate from the ternary complex. Estimates of the dissociation constant of L-lactate from the heart enzyme–NAD$^+$–lactate complex of 44 mM and 9.5 mM have been obtained (Schwert et al., 1967; Südi, 1974). Thus, although DAD$^+$ binds less tightly than NAD$^+$ to lactate dehydrogenase and has no coenzyme activity, it functions correctly in inducing the formation of a ternary complex. X-ray crystallographic studies have indicated that there may be a substantial conformational change on going from the binary to the ternary complex (Holbrook et al., 1975).

Analysis of the Labeling by [^{3}H]DAD$^+$. When tritiated DAD$^+$ was photolyzed at 350 nm in the presence of lactate dehydrogenase under the conditions specified in Table I, 16% of the available coenzyme binding sites were labeled after removal of small molecules by gel filtration. This labeling was entirely dependent upon the irradiation: less than 1% of the enzyme sites appeared to be labeled if the photolysis step was omitted (see Table I). Photolysis of the DAD$^+$ present was complete within 10 min, and the bound ligand was stable to further irradiation at 350 nm (Figure 2). Irradiation of the enzyme alone caused negligible loss of catalytic activity, and the remaining catalytic activity of photolabeled samples was commensurate with the extent of active site occupancy by [^{3}H]DAD$^+$. The dependence of the labeling on the diazirine group is shown by the facts that photolysis of the enzyme in the presence of NAD$^+$ leads to undetectable losses of catalytic activity under the conditions used and that *prephotolyzed* DAD$^+$ does not bind covalently to the enzyme upon further irradiation.

In any photoaffinity labeling study involving a macromolecular receptor and a suitably derivatized ligand, we can distinguish three possible types of labeling: (a) covalent labeling of the receptor site (specific labeling); (b) noncovalent

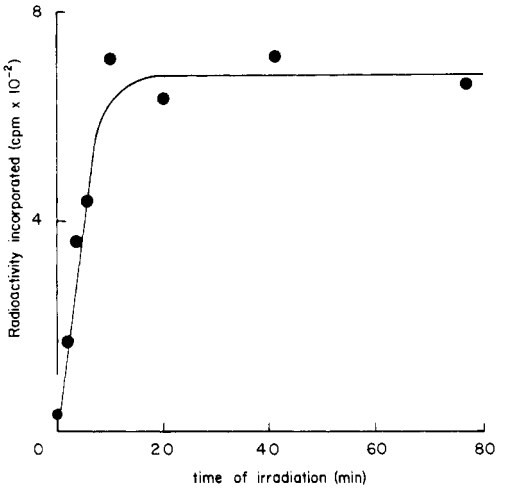

FIGURE 2: Covalent incorporation of [^{3}H]DAD$^+$ into lactate dehydrogenase as a function of the time of irradiation of the complex.

labeling, either to the receptor site or elsewhere; (c) covalent binding elsewhere on the receptor (nonspecific labeling). Generally, we seek to maximize labeling of the first type while minimizing the second and third types, but since the factors leading to each class of attachment are not independent, there is necessarily a trade-off among the three categories. For instance, a high ligand concentration will saturate the receptor site but will also result in a higher concentration of photolabile species free in solution; very tight ligand binding will favor site occupancy during photolysis but may also lead to protection of the site by the noncovalent binding of photoproducts [for further discussion, see Bayley & Knowles (1977)]. While the development of new reagents that result in shorter lived and more reactive photogenerated intermediates will help to solve some of these problems, there is undoubtedly still a need for experimental approaches that allow a clean distinction to be made between the categories cited above. These are discussed in turn.

Specific Labeling. Unless the locations of amino acids that contribute directly to a ligand binding site are known for other reasons (e.g., the hypervariable regions of the immunoglobulins could for good reasons be expected to supply the amino acid residues that constitute the hapten binding locus), there are two criteria that suggest specific receptor site labeling. These are (1) loss of receptor site function on photolabeling and (2) protection against such loss by the presence of the natural, photoinert ligand. Since loss of receptor site function may occur for reasons other than covalent photoaffinity labeling (e.g., the presence of tightly bound photoproducts or photoinactivation), protection experiments remain the most reliable way of measuring specific labeling.

NAD$^+$ binds to muscle lactate dehydrogenase with a K_{diss} of 600 μM (Stinson & Holbrook, 1973). At saturating levels of NAD$^+$ (e.g., at 50 mM NAD$^+$ and 1.4 mM DAD$^+$, only 0.5% of the enzyme sites will be occupied by DAD$^+$), the extent of photolabeling of lactate dehydrogenase by DAD$^+$ falls reproducibly from 16 to ~9% (see Table I). At high levels of NAD$^+$ (>11 mM), the extent of labeling is independent of NAD$^+$ concentration, as expected. It therefore appears that about half of the observed attachment of DAD$^+$ (in the absence of protecting NAD$^+$) is at the active site.

While ligands such as oxalate bind poorly to lactate dehydrogenase (Novoa & Schwert, 1961), the dissociation constant of oxalate from the enzyme–NAD$^+$–oxalate complex is 84 μM (Novoa et al., 1959). In the presence of oxalate (22 mM), the *effective* dissociation constant of NAD$^+$ from lactate dehydrogenase is reduced to 2.2 μM. NAD$^+$ can therefore be made to bind as tightly as NADH [K_{diss} = 3.5 μM (Stinson & Holbrook, 1973)] which cannot be used as a site-protecting ligand because of its high absorbance at 340 nm. When lactate dehydrogenase is photolabeled with DAD$^+$ after the formation of the ternary complex enzyme–NAD$^+$–oxalate, the labeling by DAD$^+$ is reduced to 3%. Oxalate alone (50 mM) has no effect on the extent of labeling, suggesting that oxalate neither binds to the enzyme–DAD$^+$ complex nor interferes with the labeling of the enzyme by the photogenerated carbene. The difference between the two protection experiments emphasizes the need for caution in interpreting raw protection data. The reason for the apparent discrepancy is explained below.

Noncovalent Binding. When labeled lactate dehydrogenase (in which 16% of the accessible sites appear to be occupied) is subjected to gel filtration in sodium dodecyl sulfate, the extent of labeling falls reproducibly from 16 to 11%. Since unphotolyzed DAD$^+$ is completely removed from the dehydrogenase by gel filtration without recourse to denaturants, it is evident that products of DAD$^+$ photolysis bind rather tightly to the enzyme. Since formation of the ternary enzyme complex with NAD$^+$ and oxalate blocks this labeling, it is apparent that the noncovalently bound material is located at the active site. These conclusions are supported by experiments in which DAD$^+$ was prephotolyzed before addition to the enzyme. Following gel filtration, 6% of the protein carried tritium label, essentially all of which was lost on gel filtration in the presence of sodium dodecyl sulfate. If prephotolyzed DAD$^+$ is added to lactate dehydrogenase containing saturating levels of NADH (3 mM) or of NAD$^+$ (38 mM) and oxalate (22 mM), less than 1% of the enzyme sites are occupied after gel filtration. This confirms that the noncovalent binding of DAD$^+$ photolysis products is at the active site.

Nonspecific Labeling. If the lifetime of the photogenerated reactive intermediate is long relative to diffusion processes, nonspecific labeling may occur. First, a species generated in free solution may live long enough to diffuse to the surface of the receptor molecule and label superficial amino acid residues at random. Second, even though the reactive species may be generated at the proper binding site, it may live sufficiently long to diffuse away and label a remote and irrelevant region of the macromolecule. To minimize these problems a short-lived species of extremely high reactivity is desirable, and one of the aims of the present study was to provide a carbene analogue of NAD$^+$ that would react more rapidly and less discriminately than, for instance, the nitrene analogue reported by Hixson & Hixson (1973). Indeed, recent comparisons of the efficacy of aryl carbenes and aryl nitrenes as lipid labeling reagents (Bayley & Knowles, 1978a,b) indicate the superiority of the photogenerated carbene for labeling chemically unreactive sites. It is clear, however, that even carbene reagents are not perfect in this respect, and we need both to estimate and to minimize the nonspecific labeling. One approach to this is to use a scavenger species (Ruoho et al., 1973).

When the active site of lactate dehydrogenase was completely blocked with NAD$^+$–oxalate, the extent of photolabeling by DAD$^+$ was ~3%. The same result was obtained by labeling the enzyme in the presence of NAD$^+$ with subsequent removal of all noncovalently bound label by gel filtration in the presence of denaturant. This residual 3% of covalently bound label appears to derive from nonspecific labeling. Since the labeling patterns are identical in either Tris or phosphate buffers (data not shown), it is evident that the amine buffer is ineffective in eliminating nonspecific la-

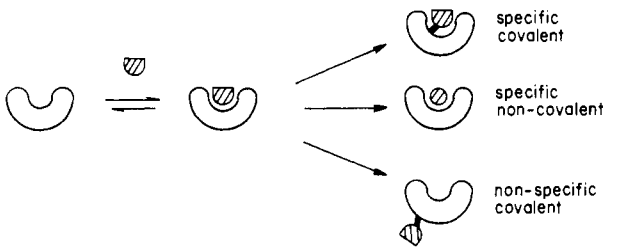

FIGURE 3: Interactions between a photolabeling reagent and its receptor.

beling. Recently, thiols have been shown to be effective scavenging reagents for aryl nitrenes (Bayley & Knowles, 1978a), and we have used thiols to eliminate nonspecific carbene labeling in the present work. When the labeling of the enzyme–NAD^+–oxalate complex is repeated in the presence of reduced glutathione (25 mM), the labeling level falls from 3 to 1%. Similarly, when lactate dehydrogenase is photolyzed in the presence of DAD^+ and glutathione, the extent of labeling drops consistently from 16 to 13%. Thus, nonspecific covalent labeling accounts for ~3% of the labeling under the conditions examined. It appears that carbenes give rise to rather little nonspecific covalent labeling (which can, in any case, be eliminated with a thiol scavenger). Although we have investigated the use of glutathione most extensively, other thiols (2-mercaptoethanol, thiolactate, and thioglycolate; see below) function equivalently.

From the experiments described, a coherent picture of the labeling by DAD^+ can be drawn (Figure 3). Photolysis of DAD^+ with lactate dehydrogenase leads to 16% labeling of the available active sites. Protection of the active site by NAD^+ alone halves this occupancy. If this labeled protein is subjected to denaturants, the labeling level falls to 3%. These facts suggest that DAD^+ labels 8% specifically at the active site and 3% nonspecifically elsewhere in the molecule and the remaining 5% of label is noncovalently bound at the active site. The three contributions to the extent of labeling are independent of one another, and the methods for eliminating each contribution are also mutually independent.

Ternary Complexes. The dependence of photolabeling efficiency upon the dissociation constant of the photolabile ligand is well documented [see, e.g., Bayley & Knowles (1977)], and the effect on the extent of photolabeling by DAD^+ within a ternary rather than a binary complex was therefore investigated. In the presence of L-lactate (50 mM) [the K_{diss} of lactate from the muscle enzyme–DAD^+ complex is 49 mM (Figure 1)], the occupancy of DAD^+ rises from 16 to 22%. After gel filtration in denaturant, the covalent labeling is found to be 18%. Experiments with [^{14}C]-L-lactate demonstrate that no lactate becomes covalently attached to the enzyme. That this increase in labeling is due to the formation of a *specific* ternary complex is shown by the effect of the substrate enantiomer D-lactate (50 mM), in the presence of which (after gel filtration in denaturant) the labeling is only 11%. Other substrates and substrate analogues (pyruvate, glyoxalate, glycolate, and mandelate) have little or no effect on the labeling of the dehydrogenase by DAD^+, and we may presume that even if specific ternary complexes are formed with these species, there is no consequential effect upon the efficacy of carbene attack on the protein.

In an attempt to eliminate the specific labeling of the active site other than by competitive protection, the effect of thiolactate and thioglycolate was studied. Thiolactate and thioglycolate are known to form ternary complexes with the enzyme and its dinucleotide cofactor, and thiolactate binds to

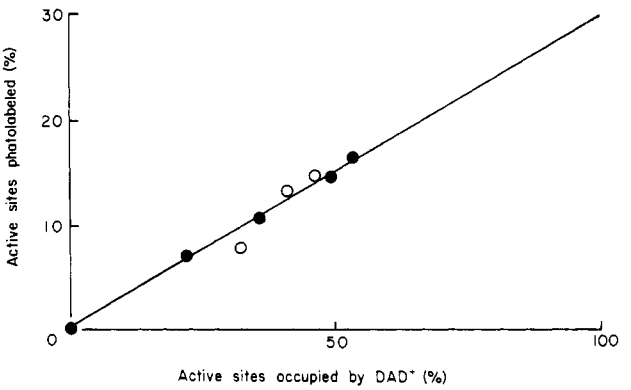

FIGURE 4: Covalent incorporation of [^{3}H]DAD^+ into lactate dehydrogenase as a function of the site occupancy before irradiation.

lactate dehydrogenase with a K_i of 3 mM (data not shown). However, neither thiolactate nor thioglycolate (at 50 mM) does more than glutathione: the labeling of enzyme is reduced only by the amount of nonspecific labeling.

To probe more fully the labeling efficiency from both the binary and ternary complexes, we performed equilibrium dialysis experiments in which the available enzyme sites were loaded to various extents with DAD^+ either by increasing the free DAD^+ concentration or by increasing the L-lactate concentration. After equilibration and irradiation, the amount of DAD^+ covalently bound was determined. The results of this experiment are summarized in Figure 4. Whether the DAD^+ is present initially in the binary complex or in the ternary complex, the extent of labeling depends only on the initial amount of DAD^+ bound. Although there may be conformational differences in the two complexes, this is not reflected by any difference in the labeling efficiency of the carbene from within these complexes. Two further points emerge from this experiment. First, by extrapolation to 100% initial occupancy of the sites, we can see that the true covalent photolabeling efficiency is ~30%. For a system with a dissociation constant of 4 mM, this represents a very acceptable efficiency of labeling. Second, even at a free DAD^+ concentration of nearly 5 mM, the nonspecific labeling does not rise out of proportion with the site-specific labeling.

Rearrangement of Diazirine to Diazo Compound. We have shown previously (Smith & Knowles, 1975) that irradiation of 3-aryl-3*H*-diazirines (in contrast to 3-aryl-3-halogenodiazirines, for example) can lead to substantial photoisomerization to the linear aryl diazomethane. It was therefore of interest to see if this species, generated by irradiation of the diazirine for an appropriate time (until, in the case of DAD^+, the A_{295} had reached its maximum), would act as an *electrophilic* affinity label for lactate dehydrogenase in the dark. A 6-min irradiation produced maximum amounts of the 3-diazo analogue of NAD^+ (the amount can be roughly estimated at 40% of the diazirine present initially), and this was then added to the enzyme in the dark. The diazo compound is stable for several hours under these conditions. Only noncovalent binding of photoproducts was detected (Table I), the amount of bound label being the same as when the prephotolysis had been carried on for 100 min, after which all diazirine *and* diazo compounds have been completely photolyzed. The presence of copper(II) during the dark incubation did not result in any reaction of diazo compound with the protein. Although the diazo compound photolyzes slowly at 350 nm, experiments in which DAD^+ was prephotolyzed for 6 min as above and then further irradiated in the presence of enzyme suggest that photolysis of the diazo compound makes essen-

tially no contribution to the covalent labeling of lactate dehydrogenase.

Other NAD^+ Analogues. A large number of analogues of NAD^+ have been synthesized (Woenckhaus, 1974; Biellmann et al., 1977), including some chemically reactive and some photolabile derivatives. In photolabeling studies, both the diazoacetate ester of the 3-(hydroxymethyl)pyridine analogue (Browne et al., 1971) and the 3-azidopyridine derivative (Hixson & Hixson, 1973) have been investigated. In the case of the [^{14}C]diazoacetate irradiated in the presence of yeast alcohol dehydrogenase, the protein fractions from a gel filtration column run under nondenaturing conditions contained amounts of radioactivity corresponding to 20% of the enzyme subunits on a molar basis (Browne et al., 1971). This number fell to ~8% in the presence of 10 mM NAD^+. These percentages represent the sum of the amounts of ligand bound covalently and noncovalently, if any (see the discussion above). When the 3-azidopyridine analogue of NAD^+ was irradiated in the presence of yeast alcohol dehydrogenase, 7% of the protein subunits contained bound label, this being reduced to 4% in the presence of NAD^+ (Hixson & Hixson, 1973). Once again, the 3% of label at the active site is presumed to comprise both covalently and noncovalently bound material. In the absence of a full discrimination among the three categories of labeling discussed above (specific covalent, nonspecific covalent, and specific noncovalent), a precise comparison with our present results cannot be made. However, from the data presented by these authors we can estimate the efficiency of covalent labeling at the active site of the dehydrogenase studied with these other reagents. In a situation where the cofacter binding site is fully occupied by the NAD^+ analogue, the labeling efficiency of the 3-azidopyridine derivative is 4%, of the 3-diazoacetate analogue is 14%, and of the 3-diazirino-substituted material used here is 21%. Finally, a photolabile analogue of NAD^+ of a quite different kind has been reported by Chen & Guillory (1977), who attached an *N*-(4-azido-3-nitrophenyl)-β-alanine residue to a hydroxyl group of the adenosine moiety of NAD^+. This material was a substrate for yeast alcohol dehydrogenase, and photolysis of the complex resulted in the attachment of 1.9 mol of label per enzyme tetramer and the concomitant loss of 91% of the enzyme's activity. Evidently this analogue is an effective photoaffinity reagent for the dehydrogenase, even though the attachment site is likely to be far removed from the catalytic locus of the enzyme.

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