Photoaffinity Labeling of D-(−)-β-Hydroxybutyrate Dehydrogenase by (Arylazido)-β-alanyl-Substituted Nicotinamide Adenine Dinucleotide†

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Received January 21, 1985

ABSTRACT: (Arylazido)-β-alanyl-substituted nicotinamide adenine dinucleotide (N_3-NAD) is a photosensitive analogue of NAD capable of photoinduced nitrene generation and insertion into a nearby molecule. In the dark, N_3-NAD can replace NAD as a cosubstrate for the mitochondrial D-(−)-β-hydroxybutyrate dehydrogenase (BDH). With purified, phospholipid-reconstituted BDH and NAD as the variable substrate, the apparent K_m and V_{max} values were respectively 0.25 mM and 62.5 μmol min^{-1} (mg of protein)$^{-1}$. With N_3-NAD as the variable substrate, these values were respectively 0.59 mM and 5 μmol min^{-1} (mg of protein)$^{-1}$. Photoirradiation of BDH in the presence of N_3-NAD resulted in irreversible inhibition of the enzyme and incorporation into the protein of radioactivity from tritiated N_3-NAD. Photoirradiation of BDH plus or minus NAD in the absence or presence of (arylazido)-β-alanine caused little or no inhibition. The photoinhibition of BDH in the presence of N_3-NAD was prevented nearly completely by addition of NADH, NAD plus β-hydroxybutyrate, or NAD plus 2-methylmalonate and partially by addition of NAD. Moreover, the presence of NADH prevented, and prior partial modification of BDH at the NAD(H)-protectable site by *N*-ethylmaleimide decreased, the incorporation of radioactivity into BDH from photoirradiated [^{3}H]N_3-NAD. The above results suggest that N_3-NAD can be used for photoaffinity labeling of BDH at the active site.

D-(−)-β-Hydroxybutyrate dehydrogenase (BDH,[1] EC 1.1.1.30) is a lecithin-requiring enzyme that is associated with the mitochondrial inner membrane. It catalyzes with ordered bi bi kinetics the reversible oxidation of D-(−)-β-hydroxybutyrate to acetoacetate in the presence of NAD as electron acceptor (Nielsen et al., 1973). The bovine heart enzyme has been reported to be tetrameric in the mitochondrial inner membrane as well as in the purified and phospholipid–reconstituted state (McIntyre et al., 1983). The monomer molecular weight is 31 500 (Bock & Fleischer, 1975). It has been shown that bovine BDH is inhibited by modifiers of mono- and dithiols (Phelps & Hatefi, 1981a,b; Fleer et al., 1984), by modifiers of arginyl residues (Phelps & Hatefi, 1981b; Fleer & Fleischer, 1983), and by ethoxyformic anhydride at pH 6.0 (Phelps & Hatefi, 1981b). Substrate protection studies have suggested that the residues modified by the above reagents might be located at or near the enzyme active site or at locations where their accessibility to modifiers is altered by substrate binding to BDH.

This paper describes the covalent modification of BDH by the photosensitive substrate analogue (arylazido)-β-alanyl-NAD (N_3-NAD). This compound was used previously for photoaffinity labeling of the NADH dehydrogenase component of complex I (NADH:ubiquinone oxidoreductase) and a mitochondrial NADH → NAD transhydrogenase (Chen & Guillory, 1981, 1984). In this study, it has been shown that in the dark N_3-NAD can replace NAD as an electron acceptor for BDH and that, when photoactivated, N_3-NAD binds covalently to the enzyme and inhibits it. Thus, it appears that N_3-NAD is a useful photoaffinity reagent for labeling the active site of BDH.

Materials and Methods

Materials. The sources of materials used were as follows: NAD and NADH from P-L Biochemicals; sodium DL-β-hydroxybutyrate, lithium acetoacetate (grade II), *N*-ethylmaleimide, dimethyl suberimidate dihydrochloride, carbonic anhydrase, ovalbumin, cross-linked bovine serum albumin, phosphorylase *a*, and β-galactosidase from Sigma; asolectin from Associated Concentrates; dimethyl pimelimidate dihydrochloride from Pierce; Hepes from Calbiochem-Behring; Tris (ultrapure) from Schwarz/Mann. (Arylazido)-β-alanyl-NAD, (arylazido)-β-[3-^{3}H]alanyl-NAD (3.8×10^7 cpm/μmol), and *N*-(4-azido-2-nitrophenyl)-β-alanine were prepared according to Chen & Guillory (1977). Apo-D-β-hydroxybutyrate dehydrogenase was purified from bovine heart mitochondria as described by Bock & Fleischer (1974).

Assay of BDH Activity. BDH activity was assayed at 37 °C by measuring the rate of NADH production at 340 nm. The standard assay mixture contained 20 mM DL-β-hydroxybutyrate, 2 mM NAD, 50 mM Hepes/Tris, pH 8.1, and a suitable amount of reconstituted BDH. The reactions were started by the addition of enzyme. Specific activity is expressed as micromoles of NADH formed per minute per milligram of protein. Protein concentration was determined by the method of Lowry et al. (1951) as modified by Peterson (1977), using bovine serum albumin as standard.

Reconstitution of the Apoenzyme with Asolectin. The purified apoenzyme (0.7–0.9 mg/mL) was incubated for 20 min at 37 °C with optimal amounts of asolectin (350–400 μg of lipid phosphorus/mg of apoenzyme) in 20 mM Tris/acetate, pH 8.0, containing 1 mM EDTA and 5 mM dithiothreitol. Enzyme activity was measured by addition of small aliquots

† Supported by U.S. Public Health Service Grants GM24887 and AM08126. This is Publication No. 3798-BCR from the Research Institute of Scripps Clinic, La Jolla, CA 92037.

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[1] Abbreviations: BDH, D-(−)-β-hydroxybutyrate dehydrogenase; NAD, nicotinamide adenine dinucleotide; N_3-NAD, (arylazido)-β-alanyl-NAD [for proposed structure, see Chen & Guillory (1977)]; [^{3}H]N_3-NAD, (arylazido)-β-[3-^{3}H]alanyl-NAD; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetate.

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(2–10 μL) of the mixture to the assay system preequilibrated at 37 °C. The apparent V_{max} of the reconstituted enzyme under the assay conditions used was 60–70 μmol min^{-1} (mg of protein)$^{-1}$. The sonicated asolectin suspension was prepared at a concentration of 900–1000 μg of phospholipid phosphorus/mL in 20 mM Tris/acetate, pH 8.0, containing 1 mM EDTA (Stiggall et al., 1977). Phospholipid concentration was estimated by phosphorus analysis (Baginski et al., 1967).

Photoaffinity Labeling of BDH. In a final volume of 100–400 μL, reconstituted BDH (0.35 mg/mL) in 10 mM potassium phosphate, pH 7.3, containing 1 mM EDTA and 20–600 μM N_3-NAD, was placed in a test tube (12 × 75 mm) and photoirradiated with a tungsten–halogen projector lamp (650 W) placed at a distance of 15 cm from the sample at the top of the test tube. To prevent heat denaturation, the test tube was placed on ice, and in cases of prolonged illumination the light was turned off after each minute of irradiation, and the sample was mixed to cool. After photoirradiation, aliquots of the enzyme were withdrawn and assayed for activity. For determination of the extent of covalent modification of BDH by N_3-NAD, (arylazido)-β-[3-^{3}H]alanyl-NAD ([^{3}H]N_3-NAD) was used. The modified BDH was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970). The gels were stained with Coomassie blue, destained, scanned for dye absorption at 560 nm, and cut into 5-mm slices. Each slice was digested at 50 °C in 0.5 mL of 30% H_2O_2, cooled, and diluted with 20 mL of Beta Blend (West Chem), and its radioactivity was determined in a Beckman scintillation counter. The concentration of N_3-NAD was estimated from its absorbance at 475 nm (ϵ_{475} = 4700 M^{-1} cm^{-1}) (Chen & Guillory, 1977).

Modification of BDH by N-Ethylmaleimide. The reconstituted BDH (0.35 mg/mL) in 10 mM potassium phosphate, pH 7.3, containing 1 mM EDTA, was incubated at room temperature with 30 μM *N*-ethylmaleimide. The reaction of BDH with *N*-ethylmaleimide was stopped at the indicated time intervals by addition of 3 mM dithiothreitol; then, aliquots of the mixture were withdrawn and assayed for enzyme activity. For photoaffinity labeling of *N*-ethylmaleimide-modified enzyme with N_3-NAD, the modified BDH was passed through Sephadex G-50 columns (5 × 70 mm) equilibrated with the same buffer as above. Photoaffinity labeling was carried out as described.

Cross-Linking of BDH. Reconstituted BDH (0.14 mg/mL) was incubated at room temperature for 1–2 h with dimethyl pimelimidate or dimethyl suberimidate (2 mg/mL) in 0.2 M triethanolamine/HCl buffer, pH 8.5 (Davies & Stark, 1970), in the absence or presence of 50 mM LiBr. Then, final concentrations of 5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 0.001% Bromphenol blue, and 10% glycerol were added, and the mixtures were placed in a boiling water bath for 3 min. Aliquots were electrophoresed at 8 mA/gel for 6 h on 5% polyacrylamide gels according to the procedure of Davies & Stark (1970).

RESULTS

Inhibition of BDH by N_3-NAD. In the dark, N_3-NAD could replace NAD as an electron acceptor for the oxidation of β-hydroxybutyrate in the presence of BDH. The double-reciprocal plot of activity vs. N_3-NAD concentration was a straight line with apparent K_m and V_{max} values of 0.59 mM and 5 μmol min^{-1} (mg of protein)$^{-1}$, respectively (data not shown). Under the same conditions, apparent K_m for NAD was about 0.25 mM, and V_{max} was 62.5 μmol min^{-1} (mg of protein)$^{-1}$. However, when N_3-NAD was added to BDH and the mixture photoirradiated as described under Materials and Methods, then BDH activity was inhibited, presumably because of nitrene generation from N_3-NAD and its covalent modification of BDH. The inhibition of the enzyme was a function of N_3-NAD concentration (Figure 1A) and the duration of irradiation (Figure 1B). Kinetic analyses (Figure 2) showed that the photodependent inhibition of BDH by N_3-NAD altered only the apparent V_{max}, not the apparent K_m values for NAD and β-hydroxybutyrate. These data indicated that photoactivated N_3-NAD results in complete inactivation of the BDH molecules with which it reacts during the course of each experiment, and that the remaining activity of the BDH/N_3-NAD mixture is due to unmodified BDH.

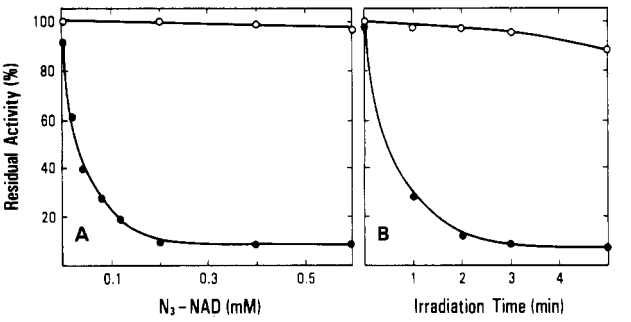

FIGURE 1: Inactivation of BDH as a function of N_3-NAD concentration (A) or of duration of photoirradiation (B). Phospholipid-reconstituted BDH at 0.35 mg of protein/mL was incubated at 0 °C in 10 mM potassium phosphate, pH 7.3, containing 1 mM EDTA. In (A) N_3-NAD was added at the indicated concentrations and the duration of photoirradiation was zero (0) and 2 min (●). In (B) 0.1 mM N_3-NAD was added only to those samples indicated by (●), and the duration of photoirradiation was varied as shown. The BDH mixtures were then sampled for activity assay as described under Materials and Methods.

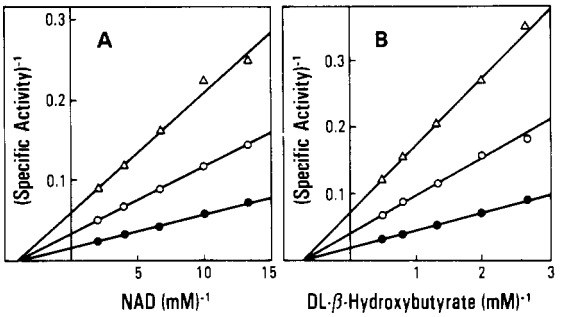

FIGURE 2: Double-reciprocal plots of the kinetics of BDH partially inhibited by photoirradiation in the presence of N_3-NAD as a function of variable concentrations of NAD (A) or β-hydroxybutyrate (B). BDH under the conditions described for Figure 1B was photoirradiated for 30 (0) or 60 s (△). The control BDH (●) was left in the dark. Then, the mixtures were sampled for activity assay at the indicated concentations of NAD (A) or β-hydroxybutyrate (B) as described under Materials and Methods.

As might be expected from the nature of the inhibitor, NADH and NAD protected BDH against inactivation by photoirradiated N_3-NAD (Figure 3, Table I), whereas β-hydroxybutyrate, acetoacetate, or the competitive inhibitor 2-methylmalonate (Tan et al., 1975) did not protect. In the presence of N_3-NAD, the latter compounds would be expected to bind to BDH and, if anything, contribute to the effectiveness of N_3-NAD to bind and inhibit the enzyme when photoactivated. At concentrations of ≥10-fold K_m, NADH (0.4 mM) was much more effective than NAD (2.5 mM) in protecting BDH against inactivation by N_3-NAD. The abortive substrate combination of NAD plus acetoacetate was not much more effective than NAD alone, but the combination of NAD plus 2-methylmalonate offered considerable protection [see also Fleer et al. (1984)]. NADH plus β-hydroxybutyrate or 2-

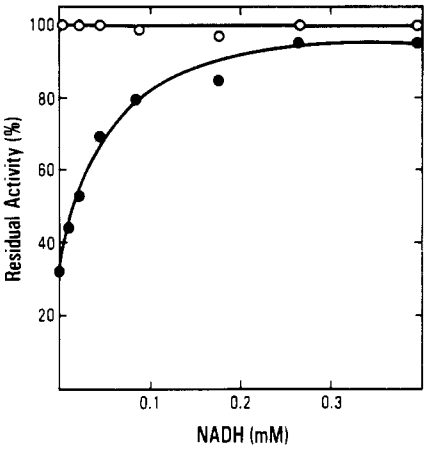

FIGURE 3: Protection by NADH of photoinactivation of BDH in the presence of N_3-NAD. BDH under the conditions described in Figure 1 was incubated in the presence of 60 μM N_3-NAD and the indicated amounts of NADH for 2 min without (0) and with (●) photoirradiation and then sampled for activity assay as described under Materials and Methods.

Table I: Effects of Substrates on Photoinactivation of BDH in the Presence of N_3-NAD[a]

additions	remaining act. (%)	additions	remaining act. (%)
none	13	NAD + acetoacetate	54
NADH	92	NAD + β-hydroxybutyrate	88
NAD	45	NAD + 2-methylmalonate	93
acetoacetate	9	NADH + acetoacetate	32
β-hydroxybutyrate	14	NADH + β-hydroxybutyrate	88
		NADH + 2-methylmalonate	91
2-methylmalonate	15		

[a] BDH under the conditions described in Figure 1 was photoirradiated for 2 min with 0.1 mM N_3-NAD and then assayed for activity. Where indicated, 0.4 mM NADH, 2.5 mM NAD, and 10 mM of other additives were also present in the mixture prior to photoirradiation. The enzyme photoirradiated for 2 min in the absence of N_3-NAD exhibited 95% of its original activity.

Table II: Effect of Photoirradiation of BDH in the Presence of N_3-NAD, NAD, (Arylazido)-β-alanine, or NAD plus (Arylazido)-β-alanine[a]

additions	remaining act. (%)	additions	remaining act. (%)
none	95	(arylazido)-β-alanine	83
N_3-NAD[b]	13	NAD + (arylazido)-β-alanine	85
NAD	97		

[a] BDH at 0.35 mg/mL in 10 mM potassium phosphate, pH 7.3, containing 1 mM EDTA was photoirradiated for 2 min and then sampled for activity assay. Where indicated, the concentration of each additive was 0.1 mM. [b] Addition of photoirradiated N_3-NAD to the assay mixture containing native BDH had no effect on the enzyme activity.

methylmalonate was as effective as NADH alone. However, the addition of acetoacetate in the presence of NADH decreased the protective effect of the latter, presumably because of oxidation of NADH to NAD, which was now present at a concentration of $\leq 1.6K_m$. NAD at 0.4 mM offered a similar degree of protection (remaining activity 27%).[2] Photoirradiation of BDH in the presence of NAD, (arylazido)-β-alanine, or NAD plus (arylazido)-β-alanine caused little or no inhibition (Table II). These results suggested that BDH inactivation is not simply due to photoirradiation in the absence or presence of NAD or modification by a nonspecific arylazide capable of nitrene generation. Rather, it appeared from the foregoing results that N_3-NAD inhibits because it binds at the BDH active site and, when photoirradiated, produces a nitrene that links covalently to BDH thereby blocking the NAD(H) binding site of the enzyme.

[2] Even though N_3-NAD absorbs light at 475 nm (Chen & Guillory, 1977), the possibility was considered that the protective effects of NADH and NAD might be due to their ability to screen light. Therefore, experiments were conducted in which mixtures of BDH and N_3-NAD were photoirradiated under the same conditions as in Table I, except that the incident light was passed through a solution of 0.4 mM NADH or 2.5 mM NAD with the same thickness as that of the reaction mixture. Placement of these light filters between the light source and the reaction mixture had no effect on the degree of inhibition of BDH by N_3-NAD.

Table III: Incorporation of Radioactivity into the Protein and Phospholipid Fractions of Reconstituted BDH after Photoirradiation in the Presence of [^{3}H]N_3-NAD[a]

sample	radioactivity (cpm) total	protein	phospholipid
reconstituted BDH	6820	4998	1425
phospholipid control	1270		1207

[a] Phospholipid-reconstituted BDH (0.14 mg of protein and 50 μg of phospholipid phosphorus) in 0.4 mL of the buffer described in Figure 1 was photoirradiated for 2 min with 0.1 mM [^{3}H]N_3-NAD (7.8×10^6 cpm/μmol) and then filtered through a Sephadex G-50 column equilibrated in the same buffer as above. An aliquot was withdrawn for determination of bound radioactivity. The remainder was concentrated in a rotary evaporator to a small volume and then 3 mL of chloroform/methanol (2:1) mixture was added and mixed. After 1 h of incubation, the sample was centrifuged for 30 min in a clinical centrifuge. The solvent containing phospholipid and the packed protein residue were separated, and the latter was dissolved in 0.1 N NaOH. Aliquots were dissolved in scintillation cocktail and their radioactivities determined in a Beckman 250 scintillation counter. The phospholipid control, at the same phospholipid phosphorus concentration as the reconstituted BDH, was treated in the same manner as above.

Table IV: Effect of Modification of BDH by *N*-Ethylmaleimide on [^{3}H]N_3-NAD Binding to the Enzyme[a]

N-ethylmaleimide treatment (s)	sp act. (μmol min^{-1} mg^{-1})	[^{3}H]N_3-NAD binding (mol/mol of BDH)
0	55	0.3
75	24.5	0.18
210	6.8	0.11

[a] Three solutions of BDH under the conditions described in Figure 1 were incubated at room temperature with 30 μM *N*-ethylmaleimide for the periods of time indicated. The interaction of BDH with *N*-ethylmaleimide was terminated by addition to each tube of 3 mM dithiothreitol, and the enzyme activities were determined. The BDH solutions were desalted by filtration through Sephadex G-50 columns equilibrated with the same buffer. The desalted enzyme was photoirradiated for 2 min in the presence of 0.1 mM [^{3}H]N_3-NAD (7.8×10^6 cpm/μmol) and then used for determination of bound radioactivity as described under Materials and Methods.

Covalent Binding of Photoirradiated [^{3}H]N_3-NAD to BDH. Photoirradiation of reconstituted BDH in the presence of [^{3}H]N_3-NAD resulted in incorporation of radioactivity into both the protein and the phospholipid fractions of the reconstituted enzyme (Table III). About 20% of the radioactivity was in the phospholipid fraction. However, control experiments suggested that labeling of the phospholipid of the reconstituted enzyme may not be specific, because a mixture containing an equivalent amount of phospholipid in the absence of protein acquired label from photoirradiated [^{3}H]N_3-NAD to the same extent (Table III). Moreover, since only about 0.01% of the total phospholipid present in the reconstituted BDH sample appeared to be labeled, addition of BDH apoenzyme to the labeled BDH/phospholipid mixture fully activated the added apoenzyme, whereas the photoinactivated BDH could not be reactivated by the addition of unmodified phospholipid (data not shown). These results indicated, therefore, that photoinactivation by N_3-NAD was due to

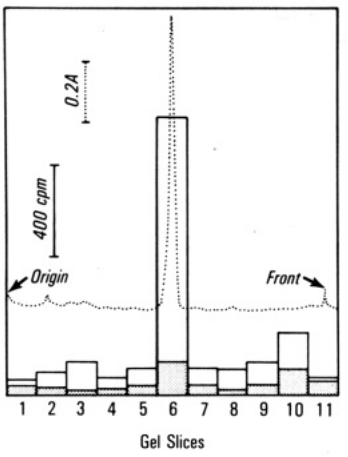

FIGURE 4: Labeling of BDH with [^{3}H]N_3-NAD. BDH under the conditions described in Figure 1 was photoirradiated for 2 min with 60 μM [^{3}H]N_3-NAD (3.8×10^7 cpm/μmol) in the absence (open bars) or presence (shaded bars) of 0.4 mM NADH. Free [^{3}H]N_3-NAD was removed by filtering BDH through Sephadex G-50 columns equilibrated in the same buffer as above. Then, aliquots of the enzyme were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed for radioactivity as described under Materials and Methods. (Dotted trace) Densitometric trace of the gel scanned at 560 nm; (bars) radioactivity of gel slices.

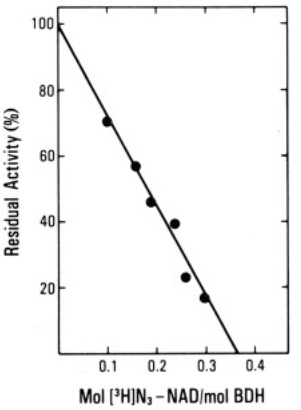

FIGURE 5: Relationship between BDH activity and its labeling with [^{3}H]N_3-NAD. Mixtures of BDH as described in Figure 1 were incubated with 100 μM [^{3}H]N_3-NAD (5.5×10^6 cpm/μmol) and photoirradiated for various lengths of time from 20 to 120 s. Aliquots of each mixture prior to and after photoirradiation were assayed for enzyme activity. The remainder of the photoirradiated samples were individually passed through Sephadex G-50 columns, equilibrated in the same buffer as above, subjected to gel electrophoresis, and analyzed for protein-bound radioactivity as described in Figure 4 and under Materials and Methods. The enzymatic activity of the samples prior to photoirradiation was taken as 100% in each case, and the molecular weight used for BDH was 32000. Similar results were obtained when photoirradiation of BDH in the presence of [^{3}H]N_3-NAD was carried out in 0.2 M triethanolamine/HCl, pH 8.5, containing 50 mM LiBr (see conditions for data of Figure 6). At 100% extrapolated activity inhibition, the extent of BDH labeling with [^{3}H]N_3-NAD was 0.36 mol/mol of BDH monomer.

modification of the BDH protein. The labeling of the protein appeared to occur at the active site, because the presence of 0.4 mM NADH during photoirradiation of the BDH/[^{3}H]N_3-NAD mixture protected 90% of BDH from becoming labeled (Figure 4). Similarly, partial inhibition of BDH by *N*-ethylmaleimide decreased the degree of incorporation of [^{3}H]N_3-NAD into the enzyme (Table IV), which is consistent with the dual facts that (a) NAD and NADH protect BDH against modification by *N*-ethylmaleimide (Phelps & Hatefi, 1981a) and (b) the K_d of NADH increases from $\sim$20 μM for the native BDH to $\sim$50 μM for the *N*-ethylmaleimide-modified enzyme (McIntyre et al., 1984).

As was mentioned earlier, BDH is considered to be tetrameric in the isolated and phospholipid-reconstituted state (McIntyre et al., 1983). It was, therefore, of interest to investigate the correlation between the degrees of N_3-NAD binding and enzyme inhibition. The results, shown in Figure 5, suggested 0.37 mol of photolyzed [^{3}H]N_3-NAD binding per mole of BDH at 100% activity inhibition. A similar stoichiometry was found at complete activity inhibition for incorporation of *N*-[^{14}C]ethylmaleimide at the NAD(H)-protectable site of the enzyme (P. V. Prasad and Y. Hatefi, unpublished results). These results suggested a situation of fraction of the sites reactivity or partial accessibility of active sites due to enzyme aggregation, which is known to occur. Thus, BDH at 0.14 mg/mL in the absence or presence of 50 mM LiBr was incubated with the cross-linking reagent dimethyl pimelimidate and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described under Materials and Methods. As seen in Figure 6, protein bands corresponding to BDH monomer (M_r 32000) and higher polymers up to hexamer were detected. There was also considerable protein at the top of the gels whose concentration increased as the incubation of BDH with dimethyl pimelimidate was prolonged, suggesting the formation of cross-linked products of higher molecular weights that were unable to penetrate the gels. These results suggested that BDH exists in solution either as a highly polymerized form or in a state of equilibrium between monomer and higher polymers. That the detection of polymerized BDH in the presence of dimethyl pimelimidate was not an artifact is supported by the fact that in the presence of dimethyl suberimidate, instead of dimethyl pimelimidate, no cross-linked products were formed. Thus, in view of the results of Figure 6, it is not possible to ascribe the stoichiometry at 100% inhibition of 0.37 mol of [^{3}H]N_3-NAD incorporation per mole of BDH to a situation of fraction of the sites reactivity [see, however, McIntyre et al. (1984) and Discussion).

Discussion

It has been shown that in the dark N_3-NAD, which is a photosensitive analogue of NAD, can serve as a cosubstrate for β-hydroxybutyrate dehydrogenase. However, when a mixture of BDH and N_3-NAD was photoirradiated, then the enzyme was inhibited. The inhibition was a function of N_3-NAD concentration and the duration of photoirradiation and was partially or completely prevented when appropriate amounts of NADH, NAD, or NAD plus 2-methylmalonate were added to the BDH/N_3-NAD mixture before photoirradiation. In the absence of N_3-NAD, photoirradiation of BDH in the absence or presence of NAD, (arylazido)-β-alanine, or NAD plus (arylazido)-β-alanine caused little or no inhibition. These results and the kinetics of partially inhibited BDH as a function of [NAD] or [β-hydroxybutyrate] suggested that the N_3-NAD inhibition involves the modification of BDH at the NAD(H) binding site by the nitrene species generated from photolysis of N_3-NAD.

The use of [^{3}H]N_3-NAD indicated that this modification involves the covalent interaction of photolyzed N_3-NAD with BDH and that 80% of the label is attached to the protein. The remainder was attached to the phospholipid fraction of the reconstituted enzyme, but control experiments suggested that the phospholipid labeling was unspecific and that it was the modification of the protein that resulted in BDH inactivation. The labeling of the protein appeared to be specific, however, because it was prevented when photoirradiation of the BDH/[^{3}H]N_3-NAD mixture was performed in the presence of 0.4 mM NADH. Furthermore, prior modification and

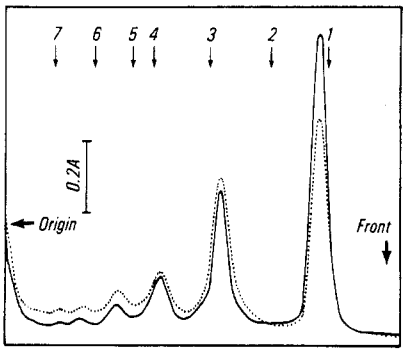

FIGURE 6: Densitometric traces of cross-linked BDH electrophoresed on sodium dodecyl sulfate–polyacrylamide gels. Phospholipid-reconstituted BDH was incubated with dimethyl pimelimidate for 60 (solid trace) or 120 (dotted trace) min in the presence of 50 mM LiBr and then subjected to gel electrophoresis as described under Materials and Methods. The gels were stained with Coomassie blue, destained, and scanned at 560 nm. Results in the absence of LiBr were essentially the same. The banding positions of molecular weight standards shown by arrows were as follows: 1, carbonic anhydrase (30 000); 2, ovalbumin (45 000); 3, bovine serum albumin (66 000); 4, phosphorylase *a* (94 000); 5, β-galactosidase (116 000); 6, bovine serum albumin dimer (132 000); and 7, bovine serum albumin trimer (198 000).

partial inhibition of BDH with *N*-ethylmaleimide decreased the degree of enzyme labeling with [^{3}H]N_3-NAD, which agreed with the facts that (a) NADH and NAD protect BDH against inhibition by *N*-ethylmaleimide (Phelps & Hatefi, 1981a) and (b) modification of the enzyme by *N*-ethylmaleimide increases the K_d for NADH by about 2.5-fold (McIntyre et al., 1984). Thus, in agreement with the inhibition results with N_3-NAD, the data for the radioactivity incorporation from [^{3}H]N_3-NAD into BDH suggest that radiolabeling of the enzyme by this reagent occurs also at the NAD(H) binding site.

Correlation of radioactivity incorporation from [^{3}H]N_3-NAD into BDH with activity inhibition suggested that at 100% inhibition only 0.37 mol of photolyzed [^{3}H]N_3-NAD is bound per mole of the enzyme. Previous work by others had indicated that isolated BDH aggregates in solution (McIntyre et al., 1978) and that the enzyme in the mitochondrial membrane or the isolated and phospholipid-reconstituted state exists as a tetramer (McIntyre et al., 1983). More recently, the same group has suggested on the basis of *N*-[^{14}C]ethylmaleimide incorporation into purified BDH that, in the presence (but not in the absence) of NAD or NADH, the enzyme exhibits half of the sites reactivity (McIntyre et al., 1984). Our data for radioactivity incorporation from [^{3}H]N_3-NAD as well as from *N*-[^{14}C]ethylmaleimide (P. V. Prasad and Y. Hatefi, unpublished results), into purified BDH, in the absence of NAD(H), indicated, however, that label incorporation at complete inhibition was less than 0.5 mol/mol of BDH. Although a situation of half of the sites reactivity remains a viable possibility, the tendency of the enzyme to aggregate in solution and thereby obscure reactive sites was an alternative that had to be considered also. Accordingly, a solution of BDH at about the same concentration as employed by ourselves and by McIntyre et al. in the above studies was treated with the cross-linking reagent dimethyl pimelimidate, and then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Cross-linked products from dimer to hexamer, plus higher aggregates that did not penetrate the gels, were detected. The possibility of half of the sites reactivity of BDH agrees with the behavior of a number of other membrane-associated enzymes [see McIntyre et al. (1984)], including the mitochondrial cytochrome *c* oxidase [for review, see Capaldi et al. (1983)], energy-linked nicotinamide nucleotide transhydrogenase (Phelps & Hatefi, 1984, 1985), and F_1-ATPase, which displays negative cooperativity with respect to [MgATP] (Cross et al., 1981; Wong et al., 1984). However the finding of aggregates of BDH under the same experimental conditions used by ourselves for photoaffinity labeling of the enzyme with [^{3}H]N_3-NAD and by McIntyre et al. (1984) for its modification by *N*-[^{14}C]ethylmaleimide suggests that protein aggregation and the resultant partial shielding of modifiable sites are possible problems that should be considered and dealt with before we can be certain of the suggested half-site reactivity of this enzyme.

Registry No. BDH, 9028-38-0; N_3-NAD, 64700-08-9; NAD, 53-84-9; NADH, 58-68-4; β-hydroxybutyric acid, 300-85-6; 2-methylmalonic acid, 516-05-2.

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