Human Aldehyde Dehydrogenase: Coenzyme Binding Studies†

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ABSTRACT: The binding of NADH and NAD^+ to the human liver cytoplasmic, E1, and mitochondrial, E2, isozymes at pH 7.0 and 25 °C was studied by the NADH fluorescence enhancement technique, the sedimentation technique, and steady-state kinetics. The binding of radiolabeled [^{14}C]NADH and [^{14}C]NAD^+ to the E1 isozyme when measured by the sedimentation technique yielded linear Scatchard plots with a dissociation constant of 17.6 μM for NADH and 21.4 μM for NAD^+ and a stoichiometry of ca. two coenzyme molecules bound per enzyme tetramer. The dissociation constant, 19.2 μM, for NADH as competitive inhibitor was found from steady-state kinetics. With the mitochondrial E2 isozyme, the NADH fluorescence enhancement technique showed only one, high-affinity binding site (K_D = 0.5 μM). When the sedimentation technique and radiolabeled coenzymes were used, the binding studies showed nonlinear Scatchard plots. A minimum of two binding sites with lower affinity was indicated for NADH (K_D = 3–6 μM and K_D = 25–30 μM) and also for NAD^+ (K_D = 5–7 μM and K_D = 15–30 μM). A fourth binding site with the lowest affinity (K_D = 184 μM for NADH and K_D = 102 μM for NAD^+) was observed from the steady-state kinetics. The dissociation constant for NAD^+, determined by the competition with NADH via fluorescence titration, was found to be 116 μM. The number of binding sites found by the fluorescence titration (n = 1 for NADH) differs from that found by the sedimentation technique (n = 1.8–2.2 for NADH and n = 1.2–1.6 for NAD^+). The binding characteristics of NADH and NAD^+ with the human mitochondrial, E2, isozyme may be rationalized on the basis of negative cooperativity induced by the bound ligand.

Two isozymes of aldehyde dehydrogenase (EC 1.2.1.3), one cytoplasmic and one mitochondrial, have been isolated from the livers of several mammalian species: sheep (Crow et al., 1974), horse (Eckfeldt et al., 1976), human (Greenfield & Pietruszko, 1977), cow (Takahashi et al., 1979; Leicht et al., 1978), and dog (Sanny, 1985). Both isozymes from human liver have been fully sequenced (Hempel et al., 1984, 1985); both are tetramers composed of identical subunits, but the subunits of the cytoplasmic isozyme have only 68% sequence homology with those of the mitochondrial isozyme. Although the primary structure of the human cytoplasmic E1 isozyme and the mitochondrial E2 isozyme has been established and X-ray crystallographic studies are now in progress, coenzyme binding studies on either isozyme were never performed. One of the reasons was the complete lack of the fluorescence (enhancement or quenching) upon binding of NADH to the cytoplasmic E1 isozyme. The mitochondrial E2 isozyme produced fluorescence enhancement upon binding NADH, but the stoichiometry was only one molecule of NADH per enzyme tetramer, and nonlinearity in Scatchard plots was observed (Pietruszko et al., 1985). Thus, the coenzyme binding studies with human aldehyde dehydrogenases required classical binding techniques.

Unlike human E1 and E2 isozymes, the analogous enzymes from sheep and horse liver showed fluorescence enhancement. Several reports from coenzyme binding studies indicated one to four binding sites per tetramer. The fluorometric titration of horse cytoplasmic F1 isozyme indicated two binding sites per tetramer (Eckfeldt & Yonetani, 1976). The binding studies of NADH and NAD^+ to sheep cytoplasmic isozyme have shown 0.7–1.5 coenzyme binding sites by fluorescence titration (MacGibbon et al., 1979; Hart & Dickinson, 1982, 1983). The fluorescence enhancement studies were then followed by other techniques, all of which yielded results indicating that the coenzyme binding site number may be less than the number of subunits in the enzyme molecule, thus suggesting "half of the sites reactivity". The studies of coenzyme binding to the human cytoplasmic and mitochondrial aldehyde dehydrogenases were initiated in the hope of obtaining better understanding of binding stoichiometry and the relationship of binding constants to the catalytic function of both isozymes.

Experimental Procedures

Materials

Nicotinamide adenine dinucleotide (NAD^+, grade I) was purchased from Boehringer-Mannheim, its reduced form (NADH) and dextran (average molecular weight 15000–20000) were obtained from Sigma Chemical Co. Radiolabeled [^{14}C]NAD^+, specific activity 260 mCi/mmol, was from Amersham. Propionaldehyde was purchased from Eastman Organic Chemicals and redistilled before use. All other chemicals were reagent grade. The cytoplasmic, E1, and mitochondrial, E2, isozymes of human liver aldehyde dehydrogenase were purified to homogeneity by the method of Pietruszko and Yonetani (1981). Prior to use, the enzymes were dialyzed against N_2-saturated 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA and 0.1% v/v 2-mercaptoethanol. Water, distilled from alkaline permanganate, was used in the preparation of all solutions.

Methods

Aldehyde Dehydrogenase Assay and Specific Activity. The enzyme was assayed by employing 1 mM propionaldehyde as substrate in 0.1 M sodium pyrophosphate buffer, pH 9.0, containing 1 mM EDTA and 500 μM NAD at 25 °C at 340 nm. The assay buffer was evacuated and nitrogenated prior to use. The rates were determined from steady-state velocity. The protein was determined by the Lowry et al. (1951) pro-

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cedure using bovine serum albumin as standard or by 280-nm absorption employing an extinction coefficient for a 1 mg/mL solution of 0.96 for the E1 isozyme and 1.05 for the E2 isozyme (Greenfield & Pietruszko, 1977).

Steady-State Kinetics. Kinetic measurements were performed employing enzyme dialyzed against 2-mercaptoethanol-free 30 mM sodium phosphate buffer, pH 6.0, where the enzyme is most stable. Activity measurements were done spectrophotometrically at 25 °C by monitoring NADH production at 340 nm in 30 mM phosphate buffer, pH 7.0, containing 1 mM EDTA. The assay buffer was evacuated at room temperature and then saturated with nitrogen. A molar absorptivity of 6.22 mM^{-1} cm^{-1} was used for NADH. The reaction rates were calculated at different, fixed concentrations of NADH. K_D values were determined from secondary plots of slope vs NADH concentration.

Enzyme Cofactor Binding Studies. Sedimentation Technique. The method of Howlett et al. (1978) was adapted for analyzing the number of binding sites with different fixed concentrations of the radioactive ligand [^{14}C]NAD^+ in 1 mL of 30 mM phosphate buffer, pH 7.0, 1 mM EDTA, and 0.1% v/v 2-mercaptoethanol at 25 °C and placed in 0.8-mL centrifuge tubes (Beckman Ultra-Clear). Dextran (2 mg/mL) was used to provide density stabilization and prevent convective stirring of the tube contents during the deceleration of the rotor. Tubes were centrifuged in the Beckman L5-65 centrifuge in an SW 50.1 rotor at $165000g_{minimum}$ for 60 min at 25 °C. The concentration of dextran and the time for sedimentation of aldehyde dehydrogenase protein were determined empirically, testing remaining aldehyde dehydrogenase activity in the upper 25 μL of solution; 10–25 μL of the top layer before and after centrifugation was assayed for radioactivity and calculated for total and free ligand concentrations, respectively. The difference in the concentration of radioactive ligand between the original solution and the supernatant was a direct measure of the amount of bound ligand. The data were treated according to the Scatchard equation for equilibrium binding (Segel, 1975):

$$\frac{\bar{\nu}}{[S]_f} = -\frac{1}{K_D}\bar{\nu} + \frac{n}{K_D} \quad (1)$$

where $\bar{\nu} = [S]_b/[E]_t$ = the ratio of the molar concentration of bound ligand to the molar concentration of enzyme, $[S]_f$ = the concentration of free ligand, K_D = the substrate dissociation constant, and n = the number of ligand binding sites per molecule of enzyme. The dissociation constant, K_D, and number of NADH and NAD^+ binding sites per molecule of aldehyde dehydrogenase were determined from the plot of $\bar{\nu}/[\text{ligand}]_{free}$ versus $\bar{\nu}$. Radiolabeled NADH was synthesized from [^{14}C]NAD^+ enzymically with yeast alcohol dehydrogenase as described by Rafter and Colowick (1957). The yield and concentration of NADH were determined spectrometrically at 340 nm using a molar absorptivity of 6.22 mM^{-1} cm^{-1} for NADH. NAD^+ was also determined spectrometrically at 340 nm, following its reduction to NADH in the presence of ethanol and yeast alcohol dehydrogenase (Ciotti & Kaplan, 1957).

Fluorescence Enhancement. The mitochondrial aldehyde dehydrogenase isozyme E2 (but not the cytoplasmic E1 isozyme) when titrated by NADH showed fluorescence emission enhancement at 425 nm, when NADH was excited at 340 nm. All fluorescence titration curves and spectra were measured at 25 °C in 30 mM phosphate buffer, pH 7.0, and 1 mM EDTA using a Perkin-Elmer MPF-2A recording spectrofluorometer. Diluted enzyme (concentration range 0.04–1 μM) was titrated in 1.5 mL of assay buffer by 10-μL additions of NADH of known concentration. Concentrations of NADH added were corrected for dilution. A control titration without enzyme in the same volume of buffer was carried out for the blank determination.

Fluorescence Charge Transfer. The dissociation constant and the number of coenzyme binding sites upon titration of E2 isozyme by NADH were also determined fluorometrically by excitation at 290 nm and emission at 425 nm.

Titration Fluorescence Competition Studies of NADH with NAD^+ and E2 Isozyme. Assuming that NAD^+ and NADH are binding to the same sites, NAD^+ was used to compete with NADH in fluorescence titration. The human cytoplasmic E2 isozyme was titrated with NADH in the presence of fixed, different concentrations of NAD^+, and then the dissociation constant of the E2–NAD^+ complex was calculated from a replot of the apparent K_D(NADH) versus [NAD^+] according to

$$K_D(\text{NADH, app}) = K_D(\text{NADH})\left[1 + \frac{[NAD^+]}{K_D(NAD^+)}\right] \quad (2)$$

Calculation of Coenzyme Binding Constants for Fluorescence Enhancement. Three methods of calculation were used to evaluate the binding data.

Method 1. Dissociation constants (D_{red}) were evaluated as described by Theorell and Winer (1959), and after checking that calculated values of D_{red} did not change significantly, we can assume that for any points on the titration curve:

$$D_{red} = \frac{[\text{enzyme}]'[\text{NADH}]'}{[\text{enzyme·NADH}]'} = \frac{[\text{enzyme}]''[\text{NADH}]''}{[\text{enzyme·NADH}]''} \quad (3)$$

and

$$C\left(\frac{a''}{b''} - \frac{a'}{b'}\right)z^2 - (a'' - a')z + (b'' - b') = 0 \quad (4)$$

where C = the aldehyde dehydrogenase binding site concentration in micronormal, a' = the sum of [NADH′], a'' = the sum of [NADH″] (single and double primes represent different points on the titration curve), b' = the sum of $(Q' - 1)$[NADH′], b'' = the sum of $(Q' - 1)$[NADH″], $z = Q - 1$, and Q' = the observed deflection divided by the blank value of NADH addition. Q was independently determined by titration of a known concentration of NADH by several additions of aldehyde dehydrogenase from the double-reciprocal plot of 1/deflection versus $1/E_t$; from eq 4, C and n were calculated.

Method 2. Assuming that the fluorescence change is directly proportional to the enzyme–coenzyme complex, the molar fraction R of the enzyme bound at each concentration of coenzyme is given by

$$R = [\text{E·NADH}]/[\text{E}]_t = \Delta F/\Delta F_{max} \quad (5)$$

where ΔF_{max} is the maximal fluorescence change with complete saturation, ΔF is the observed fluorescence change, and $[E]_t$ = the total enzyme concentration expressed in normality. ΔF_{max} can be calculated from a linear double-reciprocal plot (1/fluorescence change versus 1/[NADH]) and then K_D and NADH binding site concentration from the replot of $1/(1 - R)$ versus $[\text{NADH}]_{total}/R$ according to the Gutfreund equation (1972):

$$1/(1 - R) = [\text{NADH}]_t/K_D R - [\text{E}]_t/K_D \quad (6)$$

Method 3. The data were treated according to the Scatchard equilibrium binding eq 1, and K_D and n were determined as shown previously with the sedimentation technique. Q was independently determined as in method 1.

Table I: Constants Determined from Binding Studies of the E1 Isozyme with [^{14}C]NADH and [^{14}C]NAD$^+$ at pH 7.0 and 25 °C[a]

ligand	dissociation constant, K_D (μM)	no. of binding sites, n	ligand concn range (μM)	method
NADH	17.6 ± 3.7 (3)	1.9 ● 0.2 (3)	4–250	sedimentation
	19.2[b] (2)		0–442	steady-state kinetics
	10.3[c]			steady-state kinetics
NAD$^+$	21.4 ± 3.8 (3)	2.0 ● 0.2 (3)	3–206	sedimentation
	22.3[c]			steady-state kinetics

[a] All experiments were carried out in 30 mM phosphate buffer, pH 7.0, 1 mM EDTA, and 0.1% v/v 2-mercaptoethanol and 25 °C. Results are mean ± SD (number of experiments). [b] Value calculated from a replot reciprocal plots versus [NADH]. Activity was measured in the presence of different, fixed concentrations of NADH (0, 28, 55, 75, 110, 221, and 442 μM). [c] Value previously determined by Vallari and Pietruszko (1981).

The concentration of enzyme was determined by measurement of protein concentration using for E1 and E2 isozymes molecular weights of 219 000 and 216 000 respectively. The enzyme concentration was corrected for a maximal specific activity of 0.6 μmol of NADH min^{-1} (mg of protein)$^{-1}$ for the E1 isozyme and 1.6 μmol of NADH min^{-1} (mg of protein)$^{-1}$ for the E2 isozyme. The specific activities of the E1 and E2 isozymes used in all experiments were in the range 0.5–0.6 and 1.4–1.6 μmol of NADH min^{-1} (mg of protein)$^{-1}$, respectively.

Results

Absence of Bound Coenzyme. Homogeneous preparations of the E1 and E2 isozymes were first obtained in 1977 (Greenfield & Pietruszko, 1977). At that time, the 280-nm/260-nm ratios of the two isozymes at pH 6.0 in phosphate buffer were 1.73 and 1.67, respectively, indicating the absence of bound coenzyme. Despite this fact, additional experiments were carried out with E2 isozyme. These involved use of charcoal columns and dialysis against competing ligands including adenosine 5′-monophosphate and adenosine 5-diphosphoribose. No evidence for bound coenzyme was obtained by any of these methods.

Binding of NADH to E1 Isozyme. The binding of [^{14}C]-NADH to the human liver cytoplasmic E1 isozyme was measured by the sedimentation technique. The binding data yielded linear plots in Scatchard coordinates when 8–9.3 μM enzyme was mixed with different, fixed concentrations of [^{14}C]NADH in the range of 4–250 μM at pH 7.0 and 25 °C (Figure 1A). The average of three experiments lead to the dissociation constant K_D(NADH) = 17.6 ± 3.7 μM and a stoichiometry of n = 1.9 ± 0.2 NADH molecule bound per enzyme tetramer at pH 7.0 and 25 °C (Table I). From the steady-state kinetics, the value of the dissociation constant, K_D = 19.2 μM for NADH, as a competitive inhibitor, was calculated (Table I). The binding of NADH to the E1 isozyme did not produce any visible fluorescence enhancement in the NADH spectra at 340-nm excitation and 425-nm emission wavelength; there was also no protein fluorescence quenching.

Binding of NAD$^+$ to E1 Isozyme. When 9.3 μM enzyme was mixed with different, fixed concentrations of [^{14}C]NAD$^+$ in the range of 3–206 μM at pH 7.0 and 25 °C, linear Scatchard plots of binding data were also observed via the sedimentation technique (Figure 1B). The dissociation constant for E1 isozyme found by this technique from three separate experiments was 21.4 ± 3.8 μM, and the number of

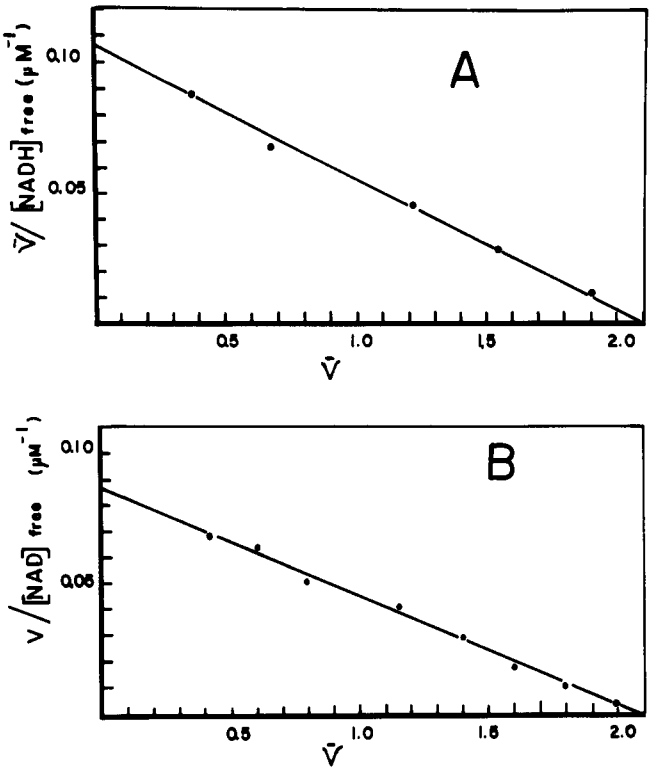

FIGURE 1: Scatchard plots for the binding of [^{14}C]NADH (A) and [^{14}C]NAD$^+$ (B) to the human cytoplasmic E1 isozyme by the sedimentation technique. 9.26 μM enzyme was mixed with different, fixed concentrations of [^{14}C]NADH (range 10–206 μM) or [^{14}C]-NAD$^+$ (range 4–150 μM) in 30 mM phosphate buffer, pH 7.0, 1 mM EDTA, and 0.1% v/v 2-mercaptoethanol at 25 °C. $\bar{\nu}$ is the ratio of the molar concentration of bound ligand to the molar concentration of enzyme. The constants obtained in this example were as follows: K_D(NADH) = 20.6 μM, n = 2.10; K_D(NAD$^+$) = 23.7 μM; n = 2.06.

binding sites per tetramer of E1 was 2.0 ± 0.2 (Table I). The dissociation constants for NAD$^+$ and NADH obtained previously by Vallari and Pietruszko (1981) from steady-state kinetics are also listed in Table I.

Binding of NADH to E2 Isozyme. The mitochondrial E2 isozyme of human liver aldehyde dehydrogenase shows fluorescence enhancement at 425-nm emission wavelength when NADH is excited at 340 nm and can be titrated fluorometrically by NADH. Fluorometric titration of a dilute enzyme solution (0.037–1 μM) with NADH (0.1–3 μM concentration) at pH 7.0 and 25 °C appears to follow a simple hyperbolic saturation isotherm (Figure 3A), and the binding data, when plotted according to eq 6 and 1, were essentially linear with the correlation coefficient in the range of 0.980–0.998 (Figures 2 and 3C). However, with higher NADH concentrations, nonlinear plots were observed which were concave toward the $1/(1 - R)$ axis and after extrapolation intersected not the $[NADH]/R$ axis but the $1/(1 - R)$ axis (method 2). Scatchard plots were also nonlinear (method 3). At low concentrations of NADH, there were no significant differences between values calculated by method 1 (D_{red} = 0.436 ± 0.090 μM, n = 1.05 ± 0.10), method 2 (K_D = 0.594 ± 0.11 μM, n = 0.90 ± 0.20), and method 3 (K_D = 0.478 ± 0.140 μM, n = 0.97 ± 0.13). The average of 10 titrations (5 titrations by method 3 and 5 other titrations calculated by methods 2 and 1) lead to the mean dissociation constant K_D(NADH) = 0.50 ± 0.13 μM and a stoichiometry of n = 0.97 ± 0.15 NADH molecule bound per E2 tetramer (Table II). The presence of chloral hydrate with concentrations up to 10 mM in the assay buffer did not change the values of K_D and n.

Table II: Constants Determined from Binding Studies of the E2 Isozyme with NADH at pH 7.0 and 25 °C[a]

dissociation constant		no. of binding sites			
K_D (μM)	obsd [NADH] range (μM)	n	obsd [NADH] range (μM)	total [NADH] range used (μM)	method
0.50 ± 0.13 (15)	0.1–3.0	0.97 ± 0.15 (15)	0.1–3.0	0.1–3.0	fluorescence enhancement
3–6 (3)[b]	4–20	0.5–1.0 (3)[b]	4–20	4–160	sedimentation
25–50 (3)[c]	20–160	1.8–2.2 (3)[c]	4–160	4.0–160	sedimentation
184 (2)[d]				0–210	steady-state kinetics

[a] All experiments were carried out in 30 mM phosphate buffer, pH 7.0, 1 mM EDTA, and 0.1% v/v 2-mercaptoethanol at 25 °C, except with the fluorescence enhancement technique where 2-mercaptoethanol was omitted from the buffer. Results are mean ± SD (number of experiments). [b] Nonlinear Scatchard plots; values estimated from the first nonlinear portion of curves. [c] Nonlinear Scatchard plots; values extrapolated from the last linear portion of curves as shown in Figure 4A. [d] Dissociation constant calculated from a replot of reciprocal plots versus [NADH]. Activity was measured in the presence of fixed, different concentrations of NADH (0, 35, 70, 140, and 210 μM).

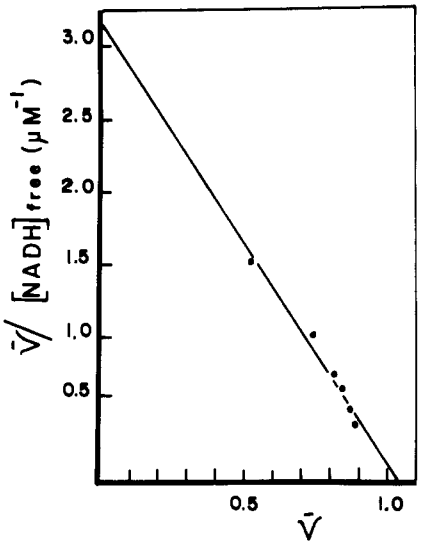

FIGURE 2: Scatchard plot for the binding of NADH to the human mitochondrial E2 isozyme by the fluorescence titration technique. 0.115 μM enzyme was titrated by single additions of 10 μL of 61 μM NADH to 1.50 mL, 30 mM phosphate buffer, pH 7.0, and 1 mM EDTA at 25 °C. Q = 36.3 inches/1 μM NADH was independently determined (see Experimental Procedures). The solution was excited at 340 nm, and the emission intensity was recorded at 425 nm. $\bar{v}$ is the ratio of the molar concentration of bound ligand to the molar concentration of enzyme. The constants obtained in this example were K_D = 0.32 μM and n = 1.03.

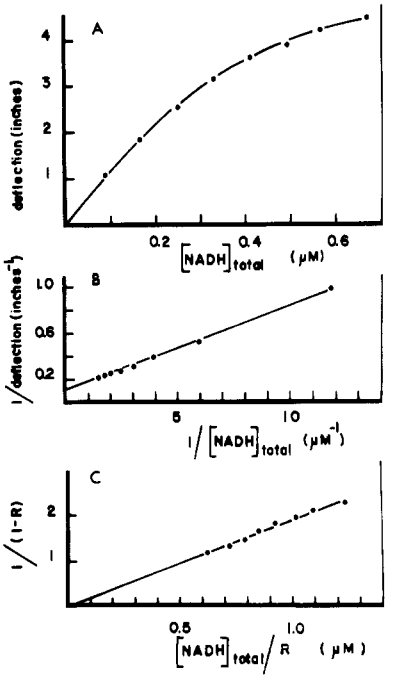

FIGURE 3: Fluorescence enhancement titration curve of NADH binding to the human mitochondrial E2 isozyme. 0.037 μM enzyme was titrated by single additions of 10 μL of 12.7 μM NADH to 1.52 mL, 30 mM phosphate buffer, pH 7.0, and 1 mM EDTA at 25 °C. The solution was excited at 340 nm, and the emission intensity was recorded at 425 nm. Total NADH concentration was corrected for dilution. R (molar fraction of the enzyme bound) and F_{max} (maximal fluorescence change at complete saturation) were determined as described in the text. (A) Direct plot; (B) double-reciprocal plot; (C) plot of $1/(1 - R)$ versus $[NADH]_{total}/R$. The constants obtained in this example were K_D = 0.53 μM, C^0 (concentration of NADH binding sites) = 0.036 μN, and n = 0.97.

When the E2 isozyme was excited at 290 nm, a protein fluorescence peak at 340 nm was observed. Titration with NADH produced only very small, ca. 20%, protein fluorescence quenching, precluding precise determination of binding data. A new charge transfer peak, however, appeared at ca. 435 nm. Titration of the enzyme at 290-nm excitation and 425-nm emission wavelengths produced a K_D value of ca. 0.30 μM and one NADH binding site per E2 tetramer (n = 0.97).

The binding of [^{14}C]NADH to the E2 isozyme was also measured by the sedimentation technique. The binding data, in the concentration range of 4–160 μM NADH used with 3.5–8.7 μM enzyme, gave nonlinear Scatchard plots, precluding precise determination of binding constants. One of the three separate experiments is shown in Figure 4A. The sedimentation technique used with higher concentrations of NADH indicated a minimum of two additional binding sites. Evaluation of the first nonlinear portion of curves gave an estimated apparent second dissociation constant of ca. 3–6 μM with an apparent number of binding sites in the range of 0.5–1.0 per E2 tetramer. Extrapolation of the last linear portion of curves indicated an apparent third dissociation constant in the range 25–50 μM and an apparent total number of binding sites in the range 1.8–2.2 per E2 tetramer (Figure 4A and Table II).

Due to the curvature of the Scatchard plots, exact values for K_D and n are impossible to determine. Depending on how the tangent is drawn, different values may be obtained. However, the second and third apparent dissociation constants determined by the sedimentation technique are ca. 1–2 orders of magnitude higher than that from the fluorescence titration technique (Table II).

The dissociation constant K_D = 184 μM for NADH determined by the steady-state kinetics is ca. 3 orders of magnitude higher than that from fluorescence titration and much higher than that found by the sedimentation technique (Table II). Constants determined from NADH and E2 isozyme binding studies are summarized in Table II.

Binding of NAD$^+$ to E2 Isozyme. The NADH fluorescence enhancement technique was used to titrate E2 isozyme with NADH in the presence of fixed, different concentrations of NAD$^+$ in the range of 0–829 μM. The binding data, when plotted according to eq 1 and 6, were essentially linear with

Table III: Constants Determined from Binding Studies of the E2 Isozyme with NAD^+ at pH 7.0 and 25 °C[a]

dissociation constant		no. of binding sites			
K_D (μM)	obsd [NAD^+] range (μM)	n	obsd [NAD^+] range (μM)	total [NAD^+] range (μM)	method
116.1 ± 18.1 (3)[b]	0–829		0–829	0–829	NADH fluorescence enhancement
5–7 (3)[c]	3–20	0.5–1.0[c]	3–20	3–85	sedimentation
15–30 (3)[d]	20–85	1.2–1.6[c]	3–85	3–85	sedimentation
102[e]					steady-state kinetics

[a] All experiments were carried out in 30 mM phosphate buffer, pH 7.0, 1 mM EDTA, and 0.1% v/v 2-mercaptoethanol and 25 °C, except with the fluoresence enhancement technique where 2-mercaptoethanol was omitted from the buffer. Results are mean ± SD (number of experiments). [b] Value calculated from plots of $1/(1 - R)$ versus $[NADH]_{total}/R$ and Scatchard plots and replot of slopes ($K_{D,app}$) versus [NAD^+]. NADH fluorescence enhancement technique was used in the presence of fixed, different concentrations of NAD^+ (range 0–829 μM). [c] Nonlinear Scatchard plots; values estimated from the first nonlinear portion of curves. [d] Nonlinear Scatchard plots; values extrapolated from the last linear portion of curves as shown in Figure 4B. [e] Value previously determined by Vallari and Pietruszko (1984a).

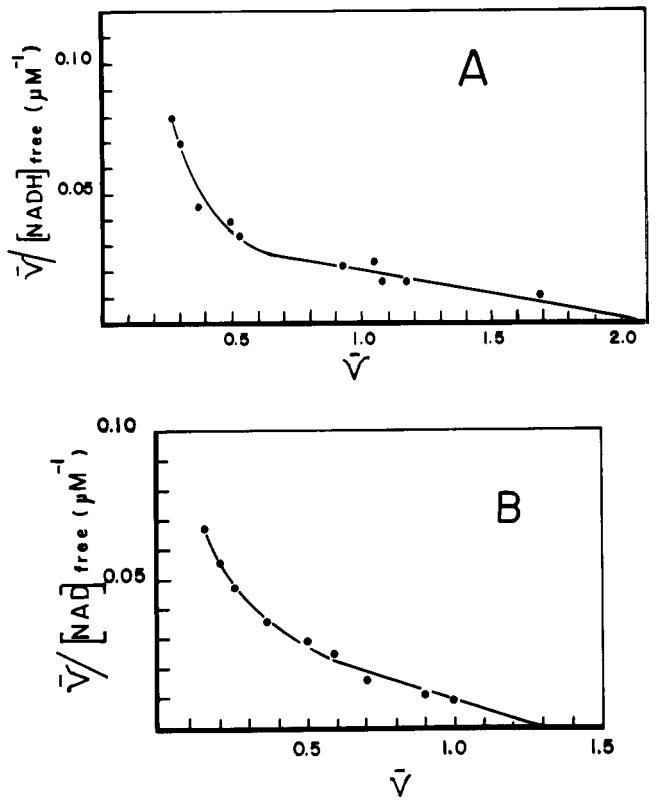

FIGURE 4: Scatchard plots for the binding of [^{14}C]NADH (A) and [^{14}C]NAD^+ (B) to the human mitochondrial E2 isozyme by the sedimentation technique. The conditions were as described in Figure 1. (A) 8.68 μM enzyme was mixed with [^{14}C]NADH (range 4–160 μM), and (B) 4.34 μM enzyme was mixed with [^{14}C]NAD^+ (range 3–85 μM). $\bar{\nu}$ is the ratio of the molar concentration of bound ligand to the molar concentration of enzyme. The estimated values of the apparent dissociation constants and the apparent number of binding sites, calculated from the first nonlinear portion of the curves, were approximately 6 μM and 0.7 for NADH and 7 μM and 0.6 for NAD^+, respectively. Those calculated from extrapolation of the last linear portion of the curves were about 50 μM and 2.1 for NADH and 30 μM and 1.3 for NAD^+, respectively.

the correlation coefficient in the range of 0.96–0.99. The dissociation constant $K_D(NAD^+)$ = 116.1 ± 18.1 μM was calculated from a replot of apparent K_D(NADH) values versus [NAD^+] (Table III).

Nonlinear Scatchard plots were observed for the binding data obtained via the sedimentation technique (Figure 4B). As with NADH binding to the E2 isozyme, this technique indicated a minimum of two binding sites for NAD^+ in the range 3–85 μM. The estimated value of the apparent dissociation constant found from the first nonlinear portion of curves from three separate experiments was ca. 5–7 μM with an apparent number of binding sites ranging from 0.5 to 1.0 per tetramer. Extrapolation from the last linear portion of curves indicated an apparent dissociation constant in the range 15–30 μM and an apparent total number of binding sites in the range 1.2–1.6 per E2 tetramer. Results of NAD^+ binding studies with the E2 isozyme are shown in Table III. The dissociation constant for NAD^+ obtained previously via steady-state kinetics by Vallari and Pietruszko (1984a) is also listed in Table III. The dissociation constant for NAD^+ from the steady-state kinetics is much higher than the values obtained by direct procedures.

Discussion

When the sedimentation technique and radiolabeled coenzymes were used, binding studies with the cytoplasmic E1 isozyme showed the dissociation constant K_D = 17.6 μM for NADH and K_D = 21.4 μM for NAD^+ (Table I). These values are in good agreement with those from steady-state kinetics, K_D = 19.2 μM for NADH (Table I), determined during this work and those previously determined, K_D = 22.3 μM for NAD^+ and K_D = 10.3 μM for NADH (Vallari & Pietruszko, 1981). Similar values have been obtained for cytoplasmic aldehyde dehydrogenases from other mammalian species. A value of the dissociation constant for NAD^+ and the sheep cytoplasmic isozyme at pH 7.0 and 25 °C was found to be 19 μM by competitive NADH fluorescence titration (Hart & Dickinson, 1983), 7 μM by equilibrium dialysis (Hart & Dickinson, 1982), 21 μM by steady-state kinetics (Hart & Dickinson, 1982), and 8 μM at pH 7.6 (MacGibbon et al., 1977) by steady-state kinetics. The dissociation constant for NAD^+ of 14.3 μM reported for the horse cytoplasmic isozyme from steady-state kinetics (Eckfeldt & Yonetani, 1976) is similar to that of the sheep cytoplasmic isozyme. NADH binding studies with horse and sheep cytoplasmic isozymes show dissociation constant values which are also in good agreement with those found here for the human cytoplasmic E1 isozyme. Thus, for the cytoplasmic isozyme from horse liver, the value of ca. 10 μM was obtained by fluorescence titration (Takio et al., 1974) and 4.6 μM by steady-state kinetics (Eckfeldt & Yonetani, 1976). For the sheep cytoplasmic isozyme, dissociation constants of 1–10 μM were obtained by fluorescence titration and also by the Sephadex equilibrium method (MacGibbon et al., 1979; Hart & Dickinson, 1983). Thus, cytoplasmic isozymes from all three species have similar dissociation constants. Moreover, the values of dissociation constants obtained by direct binding are in complete agreement with values from steady-state kinetics showing that the binding sites, detected directly, are functionally important. The values of coenzyme dissociation constants with the cytoplasmic isozymes appear to be independent of methods used for evaluation.

The Scatchard plots, from the sedimentation technique, presented in Figure 1A,B for the binding of NADH and NAD^+ to human cytoplasmic E1 isozyme are essentially linear

(there is no suggestion of any curvature in Figure 1) with stoichiometry of binding of 2 molecules of NAD^+ and 1.9 molecules of NADH per E1 tetramer (Table I). Similar values of 2 and 1.9 molecules for NADH per enzyme tetramer were found for the horse cytoplasmic isozyme by fluorescence titration and the stopped-flow technique, respectively (Eckfeldt & Yonetani, 1976). With the sheep cytoplasmic isozyme, somewhat lower values were obtained: n = 0.9–1.5 molecules/tetramer by fluorescence titration (MacGibbon et al., 1979; Hart & Dickinson, 1983), n = 0.5–0.9 by the stopped-flow technique (Hart & Dickinson, 1982), n = 0.9–1.5 by the Sephadex equilibrium method, and n = 1.2–1.5 molecules/tetramer by equilibrium dialysis (Hart & Dickinson, 1983). The results of coenzyme binding studies with the human cytoplasmic E1 isozyme showing two binding sites per enzyme tetramer are similar to those of the horse cytoplasmic isozyme and to a lesser extent to those of the sheep cytoplasmic isozyme. The cytoplasmic aldehyde dehydrogenases from human and horse liver (Hempel et al., 1984; Bahr-Lindstrom et al., 1984) consist of four identical subunits and therefore have a potential for four active sites. Thus, the fact that only two molecules of coenzyme are bound per enzyme tetramer suggests "half of the site" reactivity (McQuarrie & Bernhardt, 1971).

The available data on the coenzyme binding studies with mitochondrial aldehyde dehydrogenase from several mammalian species are far more variable than those for the E1 isozyme. A dissociation constant of 0.05 μM for NADH by the fluorescence titration and a stoichiometry of 3.8–3.9 NADH per tetramer by the fluorescence and the spectrophotometric titration were found for the sheep mitochondrial isozyme (Hart & Dickinson, 1977) and a value of the dissociation constant of 5 μM for NADH and above 100 μM for NAD^+ by the fluorescence titration for the horse mitochondrial isozyme (Takio et al., 1974). The dissociation constants, 130 μM for NADH by the kinetic measurements (Sidhu & Blair, 1975) and 0.45 μM for NADH with the stoichiometry of 0.6–0.8 NADH per tetramer by the fluorescence titration and ca. 1.7 NADH per tetramer by the stopped-flow technique (Vallari & Pietruszko, 1984b) were reported for the human mitochondrial E2 isozyme.

The human liver mitochondrial E2 isozyme can be titrated fluorometrically by NADH. Data from fluorescence titration in the small range of NADH added (Table II) gave hyperbolic binding of NADH to the E2 isozyme (Figure 4A) and linear plots with strong correlation ($r > 0.998$) in $1/(1 - R)$ versus $[NADH]/R$ and good negative correlation (r in the range −0.980 to −0.998) in Scatchard coordinates (Figure 3C and Figure 2, respectively). The binding data quantitatively evaluated by three different methods of calculation gave similar values of the dissociation constant (mean K_D = 0.50 ± 0.13 μM) and indicated only one NADH binding site per E2 tetramer (mean n = 0.97 ± 0.15, Table II). Similar values of K_D = 0.30 μM and of n = 0.97/E2 tetramer were found with charge transfer fluorescence titration. However, with higher NADH concentrations, nonlinear plots were observed.

The binding data for the NADH·E2 complex obtained here by the sedimentation technique also showed nonlinear Scatchard plots (Figure 4A). Two dissociation constants could be calculated; both were considerably higher than that from fluorescence titration. Moreover, the dissociation constant for the NADH·E2 complex calculated from steady-state kinetics was even higher than those from sedimentation and 3 orders of magnitude higher than that calculated from NADH fluorescence enhancement (Table II). Values of the dissociation constant for the NADH·E2 complex have been found to differ significantly with the technique used and the employed range of NADH concentrations. The dissociation constant changes from K_D = 0.5 μM by fluorescence titration with 0.1–3 μM NADH, via K_D = 3–6 μM and K_D = 25–50 μM by direct [^{14}C]NADH binding studied by the sedimentation technique up to K_D = 184 μM by the steady-state kinetics. The dissociation constant for NADH determined by fluorescence titration, which is much smaller than that calculated from steady-state kinetics, resembles results for glyceraldehyde-3-phosphate dehydrogenase with NAD^+ (Conway & Koshland, 1968; von Ellenrieder et al., 1972). Also, the number of binding sites, n = 1, found by fluorescence titration differs from that found by the sedimentation technique, n = 1.8–2.2 (Table II). The binding site number of the E2 isozyme is difficult to determine with certainty because of the wide range of dissociation constants. Assuming we have one binding site with a dissociation constant of 0.5 μM, one binding site with a dissociation constant of 3–6 μM, and one binding site with a dissociation constant of 25–50 μM, the fact that 184 μM dissociation constant is determined by steady-state kinetics suggests a total of four binding sites for the E2 isozyme with the site of the highest dissociation constant being catalytically active (Table IV).

Table IV: Apparent Dissociation Constants for the E2 Isozyme Complexes with NADH and NAD^+[a]

ligand	app dissociation constant (μM)			
	K_1	K_2	K_3	K_4
NADH	0.5	3–6	25–50	184
NAD^+		5–7	15–30	102[b]

[a] The dissociation constant K_1 for binding at first sites of the tetramer was determined from the fluorescence titration technique and those for two other sites, K_2 and K_3, from the sedimentation technique. The dissociation constant K_4, was estimated from steady-state kinetics. [b] The NADH fluorescence enhancement technique was used to titrate the E2 isozyme with NADH in the presence of fixed, different concentrations of NAD^+. The dissociation constant for NAD^+ calculated from a replot of K_D(NADH, app) versus $[NAD^+]$ = 116 ± 18 μM.

Data for the NAD^+·E2 isozyme complex evaluated by the sedimentation technique also gave nonlinear Scatchard plots with extrapolated values of two apparent dissociation constants for NAD^+ in the range of 5–7 and 15–30 μM and apparent total binding sites in the range of 1.2–1.6 per enzyme tetramer (Figure 4B, Table III). The binding data for NAD^+ obtained by the sedimentation technique resemble those for NADH (see above). The approximate values of the dissociation constants calculated from this procedure are almost identical for both coenzymes. Also, in the case of both NAD^+ and NADH, the dissociation constant obtained from steady-state kinetics is much larger than the values from binding experiments. The binding site described by the dissociation constant determined by the steady-state kinetics is so high that it was never saturated during binding experiments via the sedimentation technique. Dissociation constants for NAD^+ obtained via the steady-state and sedimentation techniques are listed in Table IV alongside the values obtained for NADH. Only the lowest dissociation constant for NAD^+ is not available via the above techniques. The dissociation constants for both NAD^+ and NADH (Table IV) change as saturation of the E2 isozyme is approached, increasing with the increase of saturation, a behavior consistent with either negative cooperativity or the existence of nonequivalent binding sites (Conway & Koshland, 1968; Bell & Dalziel, 1975).

It was of interest to determine the dissociation constant for NAD^+ with the E2 isozyme by competition with NADH using

NADH titration fluorescence. Unexpectedly, the calculated dissociation constant for the NAD^+·E2 complex was found to be almost identical, K_D = 116 μM (Table III), with that found from steady-state kinetics, K_D = 102 μM (Vallari & Pietruszko, 1984a). Thus, with NADH, the lowest determinable dissociation constant is from fluorescence enhancement or from charge transfer; the value obtained from the sedimentation technique is much higher. The situation is reversed for NAD^+: the highest determined value is that obtained by competition with NADH via fluorescence enhancement; the values from the sedimentation technique are much lower. With both coenzymes, the dissociation constants determined by steady-state kinetics correspond to the larger values than those determined by the direct procedures. Up to this point, the coenzyme binding data do not provide any indication of the a priori asymmetry which could also account for negative cooperativity. This apparent contradiction can be explained by a mechanism involving negative cooperativity which is induced by either NAD^+ or NADH. The E2 isozyme consists of four identical subunits (Hempel et al., 1985) and by analogy with other dehydrogenases has a potential for four equivalent catalytic sites. From our experiments, it appears that the binding sites before adding the coenzyme are all equivalent. However, binding of the first molecule of coenzyme makes it more difficult for the successive molecules to bind; the binding of coenzyme molecules inducing sequential changes in subunit conformation results in negative cooperativity. Since both NAD^+ and NADH can produce similar negative cooperativity (Table IV), during titration by NADH in the presence of NAD^+ changes in the enzyme have already been induced by NAD^+, and the sites with highest binding affinity are already occupied; only the site with the lowest affinity for NAD^+ is titrated by NADH. It is interesting to note in this connection that negative cooperativity is essential for enzyme function, since only the highest dissociation constant is catalytically important.

The mitochondrial E2 isozyme of human aldehyde dehydrogenase is in many ways more complex than the cytoplasmic E1 isozyme. This complexity, in addition to cooperative coenzyme binding, is expressed in hysteretic properties of the enzyme (Pietruszko et al., 1985). It is, therefore, conceivable that active sites with different dissociation constants for coenzymes could perform some regulatory function. As seen previously (Table I), both the dissociation constants and coenzyme binding sites are easily determinable with the E1 isozyme. Also, the determined values are in complete agreement with those from steady-state kinetics. However, even with the E1 isozyme, the binding site number is only 2, not 4, as expected from structural considerations. Thus, at the present time, it is possible that if coenzyme binding studies are extended to higher concentrations, additional binding sites may be detected.

Registry No. NADH, 58-68-4; NAD, 53-84-9; aldehyde dehydrogenase, 9028-86-8.

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