In Vitro Dissociation and Reassociation of Human Alcohol Dehydrogenase Class I Isozymes†

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ABSTRACT: Freezing (−78 °C) and thawing (25 °C) a heterodimeric human alcohol dehydrogenase class I isozyme in the presence of 0.1 M sodium phosphate/0.1 mM DTT, pH 7.0, and the subsequent separation of the scrambled isozymes by HPLC are used to prepare homodimers from heterodimers, with recovery of enzyme activity ranging from 80 to 95%. The ratio of the three isozymes obtained from a heterodimer follows the binomial distribution of 1:2:1, indicating random reassociation of the two subunits. The physical and enzymatic properties of the reassociated isozymes are the same as those obtained directly from human liver preparations. The nature of subunit–subunit interactions of human ADH class I isozymes is examined by optimizing the conditions required for the formation of the new dimers "in vitro". The effect of a number of reagents previously used in the reversible dissociation of dehydrogenases is investigated. The coenzyme NAD^+ is a potent inhibitor of the dissociation of dimers during the freeze/thaw procedure. The presence of sodium phosphate in the enzyme solution is essential during the freezing and thawing experiment. No appreciable dissociation/reassociation occurs in TES, HEPES, or even potassium phosphate. The reversible dissociation is due primarily to the decrease in pH because of the low solubility of Na_2HPO_4 at low temperatures. The reassociation occurs after thawing in a temperature-dependent process. There is no reactivation if the enzyme is incubated at 0 °C after thawing, while at 25 °C high recovery in activity is achieved in a time period ranging from 15 to 90 min. Storing heterodimer solutions (0.7–1.8 mg/mL) in the absence of coenzyme, at 4 °C, also allows the formation of the corresponding homodimers. For the $\alpha\beta_1$ isozyme, the extrapolated time for attaining the expected 1:2:1 equilibrium distribution of $\alpha\alpha$, $\alpha\beta_1$, and $\beta_1\beta_1$ dimers, respectively, is $\simeq$100 days. A monomeric form of human ADH has been isolated by gel filtration at 0 °C; however, this protein was inactive, and incubation at 25 °C only partially restored activity.

The reversible disruption of protein quaternary structure has permitted the determination of the number and type of subunits and a more profound understanding of subunit–subunit interactions for NAD(H)[1]-dependent dehydrogenases (Everse & Kaplan, 1973) and other classes of enzymes from various species (Klotz et al., 1970; Moss, 1979; Friedman & Beychok, 1979).

Studies on the lactate dehydrogenases showed that a number of factors affected the rate of hybridization during freezing and thawing. These included the presence of halides, coenzymes, buffers, and denaturing agents as well as temperature and pH (Everse & Kaplan, 1973). The present study has examined the effect of these factors on the reversible dissociation and reassociation of human alcohol dehydrogenase (ADH) class I–III isozymes with the objective of gaining insight into the nature of the forces that stabilize the quaternary structure of the enzyme. In the process, we have devised a rapid method that reversibly scrambles human ADH class I isozymes, recovering 80–95% of their activities. It makes available large amounts of homodimeric isozymes such as $\alpha\alpha$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$ which are found in human liver in very small quantities. The availability of such isozymes facilitates studies of their biochemical specificity, physiological functions, and catalytic mechanisms. Moreover, the capacity or failure to form dimers from the monomers of different ADH enzyme classes of the same and different species bears importantly on the structural and functional evolution of these various forms.

Materials and Methods

Chemicals. DTT, HEPES, and TES were purchased from Research Organics, Cleveland, OH; KP_i, NaP_i, and NaCl were from Fisher Scientific, Pittsburgh, PA; β-ME, NAD^+ (grade III), and sucrose were from Sigma Chemical Co., Milwaukee, WI. All other chemicals were of the highest quality available. Deionized–distilled water was used throughout.

Human Liver ADH Isozyme Purification. Class I–III ADH isozymes were separated and purified by previously described methods (Wagner et al., 1983, 1984; Ditlow et al., 1984), except that the enzyme preparation was first freed of NAD(H) by Affi-Gel blue affinity resin and the ion-exchange chromatographic step was accomplished by using an HPLC apparatus equipped with a SP-5PW cation-exchange column (Waters, Milford, MA) for class I and II or a DEAE-5PW anion-exchange column for class III isozymes (McEvily, unpublished results). The purified enzymes were characterized as previously reported (Bosron et al., 1979; Wagner et al., 1983; Keung et al., 1985).

Enzyme Assays. ADH activity was assayed at 25 °C by following the formation of NADH at 340 nm using a Cary

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[1] Abbreviations: ADH, alcohol dehydrogenase; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; F/T, freezing and thawing; DTT, dithiothreitol; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; β-ME, β-mercaptoethanol; P_i, inorganic phosphate; HPLC, high-performance liquid chromatography; CD, circular dichroism.

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219 spectrophotometer. The standard assay contained 33 mM ethanol and 2.5 mM NAD^+ in 0.1 M glycine/NaOH, pH 10.0. Enzyme activity units are expressed in micromoles of NADH formed per minute, based on an $\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$. Protein concentration was determined colorimetrically by a dye binding assay using bovine serum albumin as the standard (Bradford, 1976).

Spectral Measurements. Fluorescence spectra were recorded on a Perkin-Elmer MPF-3 spectrophotometer equipped with a Hitachi QPD33 recorder. CD spectra were recorded on a Cary 61 spectropolarimeter.

Generation and Separation of Scrambled Isozymes. The enzyme (≤4 mL) in 0.1 M NaP_i, pH 7.0 (±other reagents), was frozen in a dry ice/acetone mixture for 10 min and thawed at 25 °C, and activities were measured at specified time intervals. After the F/T procedure, the reassociated isozymes were concentrated and washed with 2 mM TES, pH 7.5, using Centricon 30 microconcentrators (Amicon Co., Danvers, MA) to eliminate the reagents used for the freezing and thawing steps. The samples were applied to a SP-5PW cation-exchange HPLC column equilibrated with the washing buffer. The newly formed dimers were separated by using a 0–50 mM NaCl gradient and a flow rate of 1.1 mL/min. The percentage of scrambling was determined from the area of the peaks corresponding to the various isozymes, monitoring the absorbance at 280 nm. The fractions containing ADH activity were concentrated and subjected to further characterization.

Gel Filtration of Dissociated Isozymes. The monomeric forms of human ADH class I isozymes were separated from the corresponding dimers by gel filtration at 0 °C on a Sephadex G 150-SF (15 mm × 85 cm) (Pharmacia Inc., Piscataway, NJ) column equilibrated and eluted with 50 mM NaP_i/50 mM NaCl, pH 7.5, at a rate of 5 mL/h. The column was calibrated with the molecular weight markers blue dextran 2000 (M_r 2 × 10^6), horse liver ADH (M_r 8 × 10^4), and ovalbumin (M_r 4.3 × 10^4).

Results

In order to devise a rapid and quantitative method for the preparation of homodimers from human ADH class I heterodimers, we investigated the conditions necessary for the dissociation and reassociation of such isozymes by a F/T procedure. The $\alpha\beta_1$ isozyme is used throughout all the initial studies for the optimization of the method. The conditions which proved optimal for other isozymes are then compared with those for $\alpha\beta_1$ whose activity yield and reactivation time served as the standard of reference. The previous studies on lactate dehydrogenases indicated that the choice of buffer, use of molar Cl^- ions, the presence of polyol stabilizing agents (e.g., sucrose) and sulfhydryl protecting agents (β-mercaptoethanol), and the choice of pH and temperature were all factors which affected the extent of hybridization. In particular, the presence of coenzyme often interfered with subunit dissociation (Everse & Kaplan, 1973). Therefore, unless otherwise stated, all the following experiments are performed utilizing enzyme solutions free of coenzyme.

The time during which the enzyme remains frozen proved to be unimportant: no significant difference in the recovery of activity or extent of scrambling is observed when the sample is frozen for 18 h, 5 h, 1 h, or 10 min. In the present investigation, reactivation is achieved simply by incubating the enzyme at 25 °C for 1 h. The recovery of activity is comparable to that obtained by 24-h dialysis at 4 °C. Ultrafiltration eliminates the high concentration of anions and other organic agents before the sample is loaded on the HPLC column.

Table I: Effect of Various Reagents on Isozyme F/T Technique[a]

expt	NaCl, 1.0 M	sucrose, 0.4 M	β-ME, 0.1 M	% act.[b]	% scrambling[c]
1	+	+	+	15	100
2	−	+	+	15	0
3	+	−	+	15	100
4	−	−	+	0	ND[d]
5	+	+	−	92	22
6	−	+	−	100	0
7	+	−	−	70	100
8	−	−	−	80	100

[a] All samples contained 0.2–0.4 mg/mL $\alpha\beta_1$ in 0.1 M NaP_i, pH 7.0. The appropriate F/T solution was frozen for 10 min at −78 °C and rapidly thawed at 25 °C. [b] The percentage of activity reported corresponds to the maximal recovery obtained after 60–90 min of incubation at 25 °C. The enzyme solution taken as control contained only 2 mM TES, pH 7.5. [c] The percentage of scrambling is determined by HPLC analyses as described under Materials and Methods. [d] ND, not determined; the enzyme was completely denatured.

Table II: Multiple Freezing and Thawing[a]

F/T cycle	% act.	% scrambling
1	92	22
3	92	40
5	85	70
7	80	100
9	80	100

[a] The $\alpha\beta_1$ isozyme, 0.2 mg/mL, was frozen in 0.1 M NaP_i, 1 M NaCl, and 0.4 M sucrose, pH 7.0, at −78 °C for 10 min, thawed at 25 °C in about 5 min, and then subjected to the next F/T cycle. See Table I for other conditions.

Table I shows the recovery of activity and extent of scrambling with a number of reagents previously used in F/T procedures on dehydrogenases (Chilson et al., 1965; Hart, 1971) using 10 min for freezing and ultrafiltration to remove the F/T reagents. All F/T solutions containing 0.1 M β-ME (experiments 1–4) yield only 0–15% activity when compared to the standard enzyme solution. Several washings in the ultrafiltration microconcentrator partially reversed the inhibition by β-ME but to no more than 45% of the original activity. Exclusion of β-ME increases the yield of activity. However, under such conditions, 0.4 M sucrose inhibits the scrambling process (experiments 5 and 6). A single F/T cycle for the enzyme solution containing 0.4 M sucrose and 1 M NaCl (experiment 5) results only in 22% scrambling. Multiple repetition of the F/T procedure increases the extent of scrambling which is complete after seven F/T cycles (Table II). The exclusion of both β-ME and sucrose from the F/T solution recovers 70–80% of the activity and effects complete scrambling (Table I, experiments 7 and 8). Solely freezing the enzyme in 0.1 M NaP_i, pH 7.0, recovers slightly higher activity (experiment 8). This, consequently, is the method of choice. In subsequent experiments, 0.1 mM DTT was added to protect the sulfhydryl groups of the enzyme, which results in better recovery of activity for the other isozymes. The presence of 0.5 mM NAD^+ in the F/T solution inhibits the scrambling process to less than 25%.

Kinetics of Reactivation. The reactivation of human ADH class I isozymes is markedly temperature dependent. Figure 1 shows the reactivation curve for $\alpha\beta_1$, after freezing in 0.1 M NaP_i/0.1 mM DTT, pH 7.0. Incubation of the enzyme solution at 0 °C, after thawing at 25 °C, stops the reactivation, but as soon as the sample is brought back to 25 °C, the activity increases again. The maximal recovery of activity is obtained after incubating the $\alpha\beta_1$ isozyme solution 60–90 min at 25 °C. If NAD^+, 2 mM, is added to the enzyme solution after thawing, there is no appreciable change in the rate of reac-

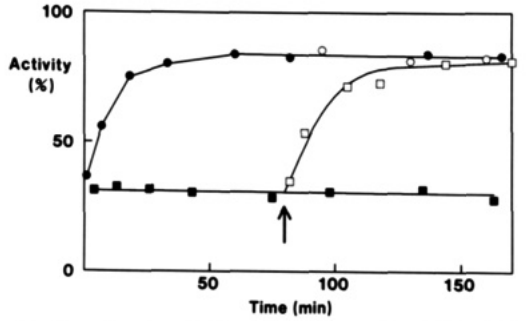

FIGURE 1: Reactivation of the $\alpha\beta_1$ isozyme after F/T. After fast thawing at 25 °C, identical portions of the enzyme solution were incubated at 0 °C (■) and 25 °C (●). At the time indicated by the arrow, identical portions of the samples incubated at 0 and 25 °C were transferred to 25 °C (□) and 0 °C (○), respectively. The enzyme concentration was 0.2 mg/mL. Activity assays were carried out as reported under Materials and Methods.

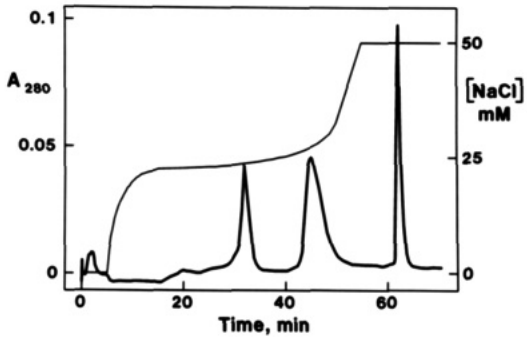

FIGURE 2: HPLC elution profiles for the isozymes generated from $\alpha\beta_1$ after the freeze/thaw procedure. Protein elution was monitored spectrophotometrically at 280 nm. The salt gradient is drawn as programmed in the gradient controller. Peak 1 centered at 32.5 min is $\alpha\alpha$, 2 at 45.5 min is $\alpha\beta_1$, and 3 at 62.7 min is $\beta_1\beta_1$.

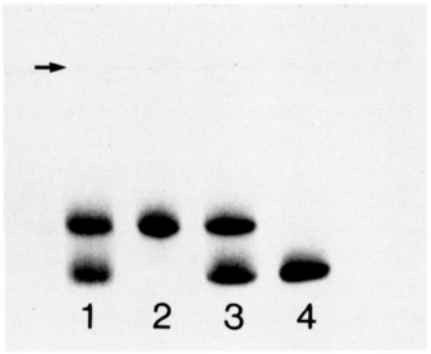

FIGURE 3: Urea–polyacrylamide gel electrophoresis of $\alpha\beta_1$ before and after the scrambling procedure. The urea gels were performed as reported by Keung et al. (1985). The origin is denoted by the arrow. Lane 1 = $\alpha\beta_1$ before F/T; lanes 2, 3, and 4 are peaks 1–3 of the HPLC chromatogram of Figure 2 corresponding to $\alpha\alpha$, $\alpha\beta_1$, and $\beta_1\beta_1$, respectively.

tivation or in the final activity yield.

HPLC Separation and Electrophoresis of Newly Formed Dimers. After complete reactivation, the enzyme mixture is subjected to HPLC to separate the individual isozymes, as reported under Materials and Methods. The $\alpha\beta_1$ isozyme generates three peaks corresponding to $\alpha\alpha$, $\alpha\beta_1$, and $\beta_1\beta_1$ in a 1:2:1 ratio, respectively (Figure 2). This is the statistically expected ratio for a random reassociation of the subunits. The identity and purity of the peaks are determined by urea gel electrophoresis (Figure 3). The same peaks are obtained when $\alpha\alpha$ and $\beta_1\beta_1$ are pooled and the F/T procedure is performed.

Effect of Buffer. The freezing and thawing of the $\alpha\beta_1$ isozyme in the presence of 0.1 M NaP_i and 0.1 mM DTT, pH 7.0, result in complete scrambling and the recovery of 70–92% of the original activity, the extent depending on the enzyme concentration (see below). The use of TES, HEPES, or even KP_i inhibits the scrambling process by more than 95%. The dissociation/reassociation process also appears to be markedly pH dependent. Attempts were made to bring about scrambling by incubation of the enzyme solution at low pH values for 3 min at room temperature before bringing the pH back to 7.0. Under these conditions, scrambling occurs below pH 5 (Table III). At pH 4.3 and 4.8, scrambling is complete, but the recovery of activity is less than that obtained by using the standard F/T procedure. At higher values of pH, 5.2 and 5.7, reasonable activity is recovered, but scrambling is incomplete. The concentration of NaP_i used in the F/T procedure also affects the degree of scrambling (Table IV). Complete scrambling occurs only at NaP_i concentrations ≥75 mM.

Table III: Incubation at Low pH[a]

pH	% act.[b]	% scrambling
3.8	0	
4.3	16	100
4.8	60	100
5.2	80	80
5.7	100	10

[a] The $\alpha\beta_1$ isozyme, 0.2 mg/mL, was incubated at room temperature for 3 min at the indicated pH in 0.1 M NaP_i and then neutralized by addition of 0.25 M NaP_i. [b] The newly formed isozymes generated by lowering the pH were unstable, and at least 75% were lost during HPLC separation.

Table IV: Sodium Phosphate Concentration Variation[a]

NaP_i concn (mM)	% act.	% scrambling
1	96	23
10	94	35
40	90	49
75	85	100
100	85	100
250	85	100

[a] The $\alpha\beta_1$ isozyme, 0.2 mg/mL, was frozen at the indicated concentration of NaP_i, pH 7.0, at −78 °C for 10 min, thawed at 25 °C, and then incubated at the same temperature to attain the maximal recovery of activity.

Table V: Enzyme Concentration Variation[a]

enzyme concn (mg/mL)	% act.	% scrambling
0.05	70	100
0.1	75	100
0.2	80-90	100
0.5	90	100
1.0	92	100

[a] The $\alpha\beta_1$ isozyme at the indicated concentration was frozen in 0.1 M NaP_i, pH 7.0, for 10 min at −78 °C, thawed, and incubated in a water bath at 25 °C for maximal recovery of activity.

Effect of Enzyme Concentration. When solutions containing less than 0.2 mg/mL are subjected to the F/T procedure, the final activity yield is lower than 80% (Table V). Under these conditions, two other bands with shorter retention times become visible in the HPLC chromatography while the peaks corresponding to the dimers are reduced considerably.

Effect of Temperature. Different methods of freezing and thawing were also examined. The time required for the complete freezing of the enzyme solutions varies from about 20 min to 5 s if freezing baths at −20 °C (ice/NaCl mixture, 3:1 v/v), −78 °C (dry ice in acetone), or −195 °C (liquid nitrogen) are used. In all cases, scrambling is 100%. At the intermediate temperature, the activity yield of reactivation for the $\alpha\beta_1$ isozyme is 75–85%, whereas at −20 and −195 °C the recoveries of activity reach only 50 and 60%, respectively. The effect

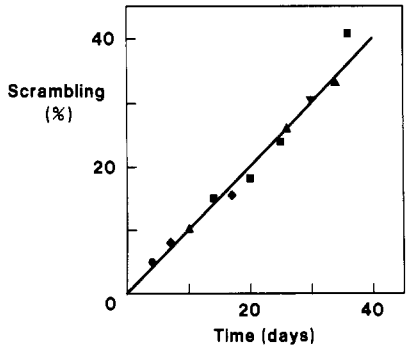

FIGURE 4: Scrambling upon storage at 4 °C of the $\alpha\beta_1$ isozyme. The percentage of scrambling was calculated from the area of the corresponding peaks in the HPLC chromatograms. The enzyme concentrations were 0.69 mg/mL for batch 1 (▲), 0.80 mg/mL for batch 2 (■), 1.04 mg/mL for batch 3 (▼), 1.63 mg/mL for batch 4 (♦), and 1.79 mg/mL for batch 5 (●). The storage buffer was 2 mM NaP_i/0.1 mM DTT at pH 7.4.

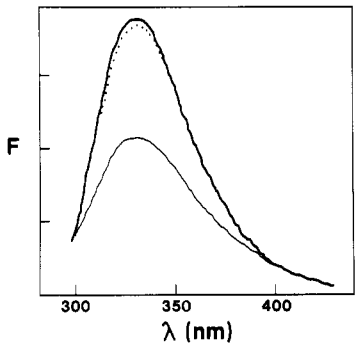

FIGURE 5: Fluorescence spectra of $\alpha\beta_1$ before F/T (thick solid line), 5 min after thawing (thin solid line), and after complete reactivation (dotted line) (90 min of incubation at 25 °C after thawing). The enzyme (0.2 mg/mL) was frozen in 0.1 M NaP_i at −78 °C for 10 min, rapidly thawed, and incubated at 25 °C.

of different rates of thawing was investigated by incubating the samples at 25 °C in a water bath (fast thawing, 4–5 min) or allowing them to stand at room temperature (slow thawing, approximately 20 min). The fast thawing procedure yields a slightly higher recovery of activity (75–85% instead of 60–70%).

Freezing of the solution is not essential to the formation of the new dimers although it greatly accelerates the process. The incubation of coenzyme-free isozyme (0.7–1.8 mg/mL) in 2 mM NaP_i/0.1 mM DTT, pH 7.4 at 4 °C, leads to scrambling of the heterodimers with formation of the corresponding homodimers as determined by HPLC and electrophoretic analyses. Forty percent scrambling of $\alpha\beta_1$ occurred after 40 days (Figure 4). It is difficult to follow the reaction for a period longer than 40 days because of the concomitant denaturation of the enzyme at 4 °C.

Detection of a Monomeric Form of Protein. The temperature dependence of the reassociation process permits the isolation of a monomeric form of the protein. After being thawed at 25 °C and being rapidly cooled to 0 °C, the enzyme mixture is applied to a gel filtration column at 0 °C. Under these conditions, two peaks with retention volumes corresponding to molecular weights of 80 000 and 40 000 are obtained. Chromatography requires 30 h to complete. The first peak, corresponding to the dimeric form, is active when assayed at 0 °C. The second one, containing the monomeric form, inactive at 0 °C, only partially regains activity after incubation at 25 °C for 3 h.

The intrinsic fluorescence and CD spectra of the enzyme preparation just after thawing also differ from those of the native enzyme. The greatest change occurs in the fluorescence of the protein (Figure 5). The enzyme species formed immediately after thawing has a marked decrease in fluorescence which is also accompanied by a 15% decrease in the negative ellipticity band, centered at 220 nm (data not shown). Only after 90-min incubation at 25 °C, when the reactivation process is complete, do the spectroscopic characteristics of the preparation become indistinguishable from those of the native enzyme.

Table VI: Optimal Conditions for the Reversible Dissociation/Reassociation of Human ADH Class I Isozymes

steps	temp (°C)	time (min)
0.2–2 mg/mL, enzyme 0.1 M NaP_i, 0.1 mM DTT, pH 7.0	21	
freezing in dry ice/acetone mixture	−78	10
thawing in water bath	25	~5
reactivation by incubn at 25 °C	25	15–90
desalting by ultrafiltration	4	~45
HPLC separation of newly formed isozymes	21	70

Table VII: Reactivation and HPLC Analysis of $\beta_1\gamma_1$ and $\beta_1\gamma_2$ after F/T Treatment[a]

	reactivation		HPLC analysis[b]		
isozyme	time[c] (min)	act. (%)	peak[d]	retention time (min)	peak area[e] (%)
$\beta_1\gamma_1$	15–20	89	1 ($\gamma_1\gamma_1$)	27.9	26.6 (1)
			2 ($\beta_1\gamma_1$)	34.5	48.8 (2)
			3 ($\beta_1\beta_1$)	62.7	24.6 (1)
$\beta_1\gamma_2$	20–30	86	1 ($\gamma_2\gamma_2$)	25.4	24.1 (1)
			2 ($\beta_1\gamma_2$)	29.9	52.0 (2)
			3 ($\beta_1\beta_1$)	63.6	23.9 (1)

[a] The enzyme (~0.25 mg/mL) was F/T as described in Table I. [b] The salt gradient used is the same as that shown in Figure 2. [c] The time represents that required for maximal recovery of activity. [d] The identity of the peak was determined by urea gel electrophoresis. [e] The number in parentheses represents the integral ratio of the peak area.

Optimal Conditions for the F/T Procedure. The procedure shown in Table VI was used for the remainder of the human class I heterodimeric isozymes. Both $\beta_1\gamma_1$ and $\beta_1\gamma_2$ are scrambled when this procedure is used. The rate of reactivation for these γ-containing isozymes is faster than that of $\alpha\beta_1$ (Table VII). Recovery of activity is maximal, about 86–89%, within 15–30 min of incubation at 25 °C. HPLC analysis indicates the presence of the respective homodimers in each case, and the ratio of the three peaks obtained is close to the binomial ratio 1:2:1.

F/T treatment of II- and χ-ADH with either $\alpha\alpha$, $\gamma_1\gamma_1$, or $\beta_1\beta_1$ does not generate new electrophoretic band(s) upon starch gel electrophoresis (results not shown), suggesting that subunits from class I ADH cannot recombine with subunits from either class II (II) or class III (χ) to form heterodimers.

Discussion

The study of the nature of the interactions that maintain the quaternary structure of enzymes and their influence on catalysis is essential for the understanding of cellular functions at the molecular level. Dissociation/reassociation experiments with protein oligomers can yield information on both the stoichiometry and molecular nature of subunit interactions. The horse EE isozyme was the first alcohol dehydrogenase to be subjected successfully to reversible dissociation "in vitro" (Drum et al., 1967). Dissociation was promoted by the addition of 8 M urea to the enzyme solution and reversed by 1:10 (v/v) dilution with an activity yield of 60–70%. Hart (1971) applied for the first time the freeze/thaw technique, developed

by Markert (1963) for studies on lactate dehydrogenases, to wheat ADH from *Trithicum*. Both of these methods were used to indicate the "dimeric" structure and subunit composition of ADH isolated from horse (Drum et al., 1967; Lutstorf & von Wartburg, 1969), *Trithicum* (Hart, 1971), and human (Smith et al., 1973; Berger et al., 1974; Bosron et al., 1983; Yin et al., 1984) sources. The success of interspecies hybridization of horse liver ADH with human $\beta_1\beta_1$ isozyme suggested structural homology between the horse and the human enzyme (Schenker et al., 1971; Berger et al., 1974) even before the amino acid sequence of the human enzyme was reported. Moreover, by carrying out the dissociation/reassociation on enzyme attached to a solid support, it was also demonstrated that the monomer of horse liver ADH is inactive (Andersson & Mosbach, 1979).

The dissociation/reassociation process has the potential of being used to prepare homodimers quantitatively from a heterodimer or vice versa. Human class I ADH exists as a number of homo- and heterodimeric isozymes which arise from the association of different types of subunits: α, β_1, β_2, β_3, γ_1, and γ_2 (Vallee, 1985). Examination of substrate specificity for the different isozymes would be easier if homodimers were readily available. However, when the conventional purification procedure is used, only small amounts of the homodimer isozymes $\alpha\alpha$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$ are usually isolated from the liver where they occur in very low concentrations. On the other hand, large quantities of the β_1-containing heterodimers can easily be purified from the liver. In the present study, the F/T technique has been employed for the production of homodimeric isozymes. The described procedure (Table VI) minimizes the time required to produce isozymes by freezing the enzyme for just 10 min and removing the various reagents rapidly by ultrafiltration. More importantly, the recovery of enzymatic activity is high. Consequently, the whole procedure can be finished within 3 h, with activity yields ranging from 80 to 95% (Table VII).

The products of reassociation are indistinguishable from the corresponding native isozymes as judged by a number of physicochemical and kinetic properties. Their retention times upon HPLC analysis, positions in starch and urea gel electrophoreses, and ethanol oxidation activity are identical with those of the native isozymes, thus confirming that the F/T procedure does not alter the biochemical properties of the enzyme.

The hybridization of oligomers can usually occur by either of two fundamentally different pathways: the protein dissociates to monomers and then reassociates, or one subunit is displaced by interaction with another oligomer without intermediate formation of monomers. In the present study, the fluorescence (Figure 5) and CD spectra clearly show that the enzyme's secondary and tertiary structures are altered in the freezing and thawing process. The change in protein fluorescence may be due to a different environment of Trp-314 which is considered to be buried in a hydrophobic environment at the intersubunit interface in the horse enzyme (Brändén et al., 1975; Eftink, 1986). The spectroscopic features of the reconstituted isozyme become indistinguishable from those of the native enzyme only after complete reactivation. Furthermore, the success in separating the monomers by gel filtration at low temperature demonstrates that under the conditions used, subunit dissociation is likely the principal pathway for the "in vitro" hybridization of human ADH class I isozymes.

The subunits of human ADH are likely held together by a number of different types of intermolecular forces including hydrogen bonds, hydrophobic interactions, and ionic interactions. The results of the present study suggest that the alteration of ionic and hydrophobic forces and removal of the coenzyme are all crucial to the dissociation/reassociation process.

The critical role of ionic interactions in subunit dissociation is reflected in the choice of buffers used for the scrambling experiment. Thus, complete scrambling occurs for NaP_i, while <5% scrambling occurs for KP_i, TES, or HEPES buffers. The radically different response to change in temperature of the Na but not KP_i buffer in the F/T procedure suggests that a change in pH is critical to the dissociation/reassociation process. During the freezing process, the $NaH_2PO_4/Na_2HPO_4/H_2O$ system (pH 7.0 at 25 °C) approaches pH 3.6 near its eutectic point (−9.9 °C) whereas the KP_i solution increases the pH during cooling (pH 7.5 near the eutectic point, −16.7 °C) (van den Berg, 1959; van den Berg & Rose, 1959). The results of the effect of pH on the extent of scrambling also support the hypothesis that subunit dissociation may occur as a result of a rapid pH drop during the freezing process (Table III). Low pH has also been reported to cause dissociation of a number of dehydrogenases (Chilson et al., 1965, 1966; Jaenicke, 1974).

The finding that lowering the temperature accelerates subunit dissociation is indicative of the requirement of weakening of hydrophobic forces. This is also supported by the fact that the rate of reactivation is increased by increasing the temperature (Figure 1). Subzero temperatures are not essential to the formation of the new dimers, though it greatly accelerates the process. A slow scrambling process was also observed after storing coenzyme-free $\alpha\beta_1$ in 2 mM NaP_i/0.1 mM DTT, pH 7.4, at 4 °C for a long period of time (Figure 4).

The coenzymes NAD^+ and NADH have been reported to affect the dissociation/reassociation process of several dehydrogenases in different ways. For example, NAD^+ binding has been reported to inhibit dissociation of several lactate dehydrogenases (Everse & Kaplan, 1973) and prevent dissociation of pig heart mitochondrial malate dehydrogenase, even at concentration as low as 10^{-8} N (Shore & Chakrabarti, 1976). It has also been suggested that the coenzyme increases the rate of reassociation of pig heart mitochondrial malate dehydrogenase (Wood et al., 1981); it increases the yield of reassociation of yeast glyceraldehyde-3-phosphate dehydrogenase (Rudolph et al., 1977a) and pig heart/muscle lactate dehydrogenase (Rudolph et al., 1977b).

In the present investigation, NAD^+ (2 mM) has a negligible effect on the reassociation process by the F/T procedure while the presence of 0.5 mM NAD^+ inhibits the scrambling during the dissociation process. Upon coenzyme binding, the horse enzyme undergoes large conformational changes which may stabilize the quaternary structure; they would likely retard subunit dissociation (Eklund & Brändén, 1987). The fact that the class I ADH scrambling process is accomplished much more readily without coenzyme present may therefore reflect similar influences of the coenzyme on stabilizing the human ADH quaternary structure. This is a particularly fortuitous situation since preparation of homodimers in the absence of coenzyme facilitates kinetic studies of the isozymes, especially those concerned with coenzyme association and dissociation.

Primary sequence analyses of all the subunits of human liver ADH have been performed (Jörnvall et al., 1987a,b; Kaiser et al., 1988). The various class I subunits are 90% homologous with one another. The class II (π) and class III (χ) subunits are only about 60% homologous with the class I subunits. This

structural difference likely influences the different catalytic properties of the different classes of isozymes (Vallee, 1985; Bosron & Li, 1987). On the basis of the structure of horse liver ADH obtained by X-ray crystallography (Brändén et al., 1973, 1975), the sequence from amino acid residues 270–330 is the main segment involved in subunit interaction. Within this region, the various class I subunits have exactly the same amino acid composition at 54 of these positions and vary in structure only slightly at 7 variable positions (Jörnvall et al., 1987a). This likely reflects the ease by which the class I subunits can reassociate randomly after dissociation (Table VII). However, large differences in this subunit interaction region exist between both the class II (Π) and the class III (χ) ADH when compared with each other and the class I subunits. Thus, when the class II and class III enzymes are compared to the class I $\beta_1\beta_1$ enzyme, there are respectively only 18 and 20 identical amino acids out of the total of 61 (Kaiser et al., 1988). This fact likely accounts for the apparent lack of cross-hybridization between Π- or χ-ADH with class I subunits. Computer modeling (Eklund, personal communication) of the various subunits also confirms that interclass subunits are structurally incompatible.

Beyond the possible insights on the nature of the forces involved in oligomer stabilization, we have developed a rapid and quantitative method for the formation of in vitro dimers indistinguishable from those which occur in the native state that can be used for further physicochemical characterization and substrate specificity studies of the human ADH isozymes.

Registry No. ADH, 9031-72-5; NAD, 53-84-9; TES, 7365-44-8; HEPES, 7365-45-9; β-ME, 60-24-2; KP_i, 16068-46-5; NaP_i, 7632-05-5; NaCl, 7647-14-5; sucrose, 57-50-1.

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