Bosron, W. F., & Li, T.-K. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 921.
Bosron, W. F., Li, T.-K., Dafeldecker, W. P., & Vallee, B. L. (1979a) *Biochemistry 18*, 1101–1105.
Bosron, W. F., Li, T.-K., & Vallee, B. L. (1979b) *Biochem. Biophys. Res. Commun. 91*, 1549–1555.
Bosron, W. F., Li, T.-K., & Vallee, B. L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5784–5788.
Bosron, W. F., Crabb, D. W., & Li, T.-K. (1982) *Alcohol.: Clin. Exp. Res. 6*, 291.
Bühler, R., & von Wartburg, J.-P. (1982) *FEBS Lett. 144*, 135–139.
Burger, A. R., & Vallee, B. L. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1886.
Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104–137.
Cleland, W. W. (1979) *Methods Enzymol. 63A*, 103–138.
Dubied, A., von Wartburg, J.-P., Bohlken, D. P., & Plapp, B. V. (1977) *J. Biol. Chem. 252*, 1464–1470.
Giblett, E. R. (1976) *Cytogenet. Cell Genet. 16*, 65–74.
Harada, S., Agarwal, D. P., & Goedde, H. W. (1978) *Hum. Genet. 40*, 215–220.
Hart, G. E. (1971) *Mol. Gen. Genet. 111*, 61–65.
Lange, L. G., & Vallee, B. L. (1976) *Biochemistry 15*, 4681–4686.
Li, T.-K., Bosron, W. F., Dafeldecker, W. P., Lange, L. G., & Vallee, B. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4378–4381.
Pietruszko, R., Theorell, H., & De Zalenski, C. (1972) *Arch. Biochem. Biophys. 153*, 279–293.
Schenker, T., Teepel, L., & von Wartburg, J.-P. (1971) *Eur. J. Biochem. 24*, 271–279.
Smith, M., Hopkinson, D. A., & Harris, H. (1971) *Ann. Hum. Genet. 34*, 251–271.
Smith, M., Hopkinson, D. A., & Harris, H. (1973) *Ann. Hum. Genet. 36*, 401–414.
Strydom, D. J., & Vallee, B. L. (1982) *Anal. Biochem. 123*, 422–429.
Theorell, H., & Chance, B. (1951) *Acta Chem. Scand. 5*, 1127–1144.
Vallee, B. L., & Bazzone, T. J. (1983) in *Isoenzymes: Current Topics in Biological and Medical Research* (Rattazzi, M. C., Scandalios, J. G., & Whitt, G. S., Eds.) Vol. 8 (in press).
Whitehead, E. P. (1978) *Biochem. J. 171*, 501–504.
Wratten, C. C., & Cleland, W. W. (1963) *Biochemistry 2*, 935–941.
Yoshida, A., Impraim, C. C., & Huang, I.-Y. (1981) *J. Biol. Chem. 256*, 12430–12436.

Kinetic Properties of Human Liver Alcohol Dehydrogenase: Oxidation of Alcohols by Class I Isoenzymes†

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ABSTRACT: Class I isoenzymes of alcohol dehydrogenase (ADH) were isolated by chromatography of human liver homogenates on DEAE-cellulose, 4-[3-[*N*-(6-aminocaproyl)-amino]propyl]pyrazole–Sepharose and CM-cellulose. Eight isoenzymes of different subunit composition ($\alpha\gamma_2$, $\gamma_2\gamma_2$, $\alpha\gamma_1$, $\alpha\beta_1$, $\beta_1\gamma_2$, $\gamma_1\gamma_1$, $\beta_1\gamma_1$, and $\beta_1\beta_1$) were purified, and their activities were measured at pH 10.0 by using ethanol, ethylene glycol, methanol, benzyl alcohol, octanol, cyclohexanol, and 16-hydroxyhexadecanoic acid as substrates. Values of K_m and k_{cat} for all the isoenzymes, except $\beta_1\beta_1$-ADH, were similar for the oxidation of ethanol but varied markedly for other alcohols. The k_{cat} values for $\beta_1\beta_1$-ADH were invariant ($\sim$10 min^{-1}) and much lower (5–15-fold) than those for any other class I isoenzyme studied. K_m values for methanol and ethylene glycol were from 5- to 100-fold greater than those for ethanol, depending on the isoenzyme, while those for benzyl alcohol, octanol, and 16-hydroxyhexadecanoic acid were usually 100–1000-fold lower than those for ethanol. The homodimer $\beta_1\beta_1$ had the lowest k_{cat}/K_m value for all alcohols studied except methanol and ethylene glycol; k_{cat} values were relatively constant for all isoenzymes acting on all alcohols, and, hence, specificity was manifested principally in the value of K_m. Values of K_m and k_{cat}/K_m revealed for all enzymes examined that the short chain alcohols are the poorest while alcohols with bulky substituents are much better substrates. The experimental values of the kinetic parameters for heterodimers deviate from the calculated average of those of their parent homodimers and, hence, cannot be predicted from the behavior of the latter. Thus, the specificities of both the hetero- and homodimeric isoenzymes of ADH toward a given substrate are characteristics of each. Ethanol proved to be one of the "poorest" substrates examined for all class I isoenzymes which are the predominant forms of the human enzyme. On the basis of kinetic criteria, none of the isoenzymes of class I studied oxidized ethanol in a manner that would indicate an enzymatic preference for that alcohol.

Interest in alcoholism as a metabolic disease has intensified the quest for basic knowledge of the enzyme(s) responsible for the oxidation of ethanol. Blair & Vallee (1966) first demonstrated that the alcohol dehydrogenase (EC 1.1.1.1) (ADH)[1] of human liver is a mixture of isoenzymes, and subsequently more than a dozen isoenzymes of human liver ADH have been recognized (Smith et al., 1973; Bosron et al., 1979a, 1980; Pares & Vallee, 1981). All of the purified ADH isoenzymes of human liver are dimeric proteins composed of

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[1] Abbreviations: ADH, alcohol dehydrogenase; 16-HHA, 16-hydroxyhexadecanoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; CapGapp, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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subunits of 40 000 daltons and contain 4 g-atoms of Zn^{2+}/80 000 daltons (Bosron et al., 1979a; Lange & Vallee, 1976; Pares & Vallee, 1981).

Human liver ADH isoenzymes can be differentiated into three classes based on their physical and enzymatic properties (Strydom & Vallee, 1982; Vallee & Bazzone, 1983). Class I[2] includes all of the isoenzymes which migrate cathodically on electrophoresis at pH 8.2 and are inhibited by 4-methylpyrazole with a $K_i \simeq 0.2$ μM (Li & Theorell, 1969). This mixture of purified isoenzymes of ADH is isolated conveniently as a class by affinity chromatography using CapGapp as an immobilized ligand (Lange & Vallee, 1976; Lange et al., 1976). Hence, class I includes—but is not necessarily limited to—the isoenzymes of ADH described by Smith et al. (1973) which are composed of α, β_1, β_2, γ_1, or γ_2 subunits. Class II refers to ADH isoenzymes which are cathodic but have a lower electrophoretic mobility at pH 8.2 than class I isoenzymes. π-ADH, the principal class II isoenzyme, is inhibited much less effectively by 4-methylpyrazole (K_i = 2 mM) than are the class I isoenzymes and therefore is not retained by CapGapp–Sepharose (Bosron et al., 1979a). The class III isoenzymes (χ-ADH) are virtually not inhibited at all by 4-methylpyrazole and migrate anodically on electrophoresis at pH 8.2 (Pares & Vallee, 1981).

Kinetic studies of these three classes of enzymes have suggested that their substrate specificities differ (Burger & Vallee, 1981; Vallee & Bazzone, 1983). While the isoenzymes of all these classes oxidize ethanol, this substrate does not saturate χ-ADH (class III) at concentrations as high as 2 M. Moreover, the K_m of π-ADH toward ethanol (33 mM) (Bosron et al., 1979a) is much higher than that of the unresolved class I isoenzymes (2 mM) (Lange et al., 1976). Neither class II (Bosron et al., 1979a) nor class III ADH isoenzymes oxidize methanol (Vallee & Bazzone, 1983), but they do oxidize a variety of other alcohols by apparent Michaelis–Menten kinetics.

The isoenzymes of class I, which are readily purified as a group by affinity chromatography (Lange & Vallee, 1976), have been studied most extensively. Collectively, they exhibit broad specificity; they catalyze the oxidation of a great variety of alcohols, including primary aliphatic (Lange et al., 1976) and aromatic alcohols, diols (Blair & Vallee, 1966), ω-hydroxy fatty acids (Burger & Vallee, 1981), and sterols including digitoxin and digitoxigenin (Frey & Vallee, 1979, 1980). They also reduce the corresponding broad range of aldehydes and ketones (Blair & Vallee, 1966; Lange et al., 1976). Even though the kinetic properties of class I isoenzymes toward numerous substrates are known, this knowledge is of limited value since it represents the average of the action of the constituent isoenzymes which may vary in number, relative amounts, and possibly specificities in preparations isolated from different human livers. Thus, the characterization of kinetic properties and substrate specificities of the purified isoenzymes of ADH is essential to the recognition of their individual metabolic functions. We here report the results of such a substrate specificity study of the individual class I ADH isoenzymes. A preliminary account has been presented (Burger & Vallee, 1981).

Experimental Procedures

Materials. NAD^+ (grade III and grade AA1), Tris (reagent grade), and 16-HHA were purchased from Sigma Chemical Co., St. Louis, MO, and ion-exchange resins from Whatman Chemical Separation, Clifton, NJ. Ethanol (100%) was obtained from U.S. Industrial Chemical Corp., New York, other alcohols were from Fisher Scientific, Medford, MA, and all were used without further purification.

Purification of Class I ADH Isoenzymes.[3] Human livers were obtained at autopsy within 12 h postmortem and were stored at −70 °C. Class I ADH isoenzymes were isolated by procedure of Lange & Vallee (1976) using 1 mM NAD^+ in 50 mM sodium phosphate buffer, pH 7.5. The unresolved mixture of isoenzymes was then concentrated to 10–20 mL on an Amicon ultrafiltration membrane (UM-50) and dialyzed against 1 L of 5 mM Tris–phosphate, pH 7.7, containing 1 mM NAD^+. The pH of all solutions was adjusted at 4 °C.

Dialyzed samples were added to a column (2.5 × 40 cm) of CM-cellulose (CM-52) equilibrated with 5 mM Tris–phosphate, pH 7.7, containing 1.0 mM NAD^+. Elution was performed with 200–500 mL of Tris–phosphate, 5 mM, containing 1.0 mM NAD^+, pH 7.7, followed by a 1500-mL linear gradient of 0–30 mM NaCl in the same buffer. Ten-milliliter fractions were collected and assayed for enzymatic activity and protein content. Seven distinct peaks of activity and protein were generally obtained, and each was concentrated to about 10 mL as before followed by dialysis against 5 mM sodium phosphate–1 mM NAD^+, pH 7.2. Individual fractions were rechromatographed on a column (1.6 × 25 cm) of CM-cellulose (CM-52) equilibrated with 5 mM sodium phosphate–1 mM NAD^+, pH 7.2. The column was eluted with at least 50 mL of sodium phosphate (5 mM)–1 mM NAD^+, pH 7.2, followed by a 500-mL linear gradient of 0–20 mM NaCl in the same buffer. Five-milliliter fractions were collected. On the basis of assays for enzymatic activity and protein concentration, fractions were pooled and concentrated to about 10 mL as before.

Enzyme Assays. ADH activity was measured as the increase in absorbance at 340 nm due to the oxidation of alcohols in the presence of 2.4 mM NAD^+ in 0.1 M glycine buffer, pH 10.0 (Lange et al., 1976). One unit of ADH activity is defined as the amount of enzyme required to produce 1 μmol of NADH/min at 25 °C. All assays to monitor columns and to assess enzyme activity were performed with 33 mM ethanol.

Electrophoretic Procedures. Starch gel electrophoresis was performed according to Bosron et al. (1979b) and gels (11 × 24.5 × 0.5 cm) were run at pH 8.2, at 720 V, and for 6 h. Samples (15–30 μL) of a solution containing 0.5–1.4 units/mL were applied to paper wicks (9 × 1.5 mm) and inserted into the gel at the center of the slab. Immediately after electrophoresis, the gels were sliced longitudinally to yield four slabs. The two center slabs (0.5–0.7 mm thick) were stained for ADH activity with 200 mL of either 100 mM ethanol or 100 mM pentanol containing 50 mM sodium pyrophosphate, 0.6 mM MTT, 0.26 mM PMS, and 1.2 mM NAD^+, pH 8.6. Gel slabs were incubated at 45 °C for 10–15 min. After being stained, the gels were washed with water and photographed.

Protein Determination. Protein concentration was determined colorimetrically either by the method of Lowry et al. (1951) or using the Bio-Rad protein assay (*Bio-Rad Technical Bulletin*, 1978). In both cases bovine serum albumin was the standard. The validity of these estimations was determined by measuring the UV-absorption spectrum of class I iso-

[2] Class I ADH isoenzymes are electrophoretically cathodic proteins (at pH 8.2) inhibited by micromolar concentrations of 4-methylpyrazole and include the homo- and heterodimers composed of α, β_1, β_2, γ_1, and γ_2 monomers.

[3] The purification procedure employed here is modified from that developed and recommended to us by Dr. T.-K. Li, Departments of Medicine and Biochemistry, Indiana University Medical School. We greatly appreciate his communication of pertinent data prior to publication.

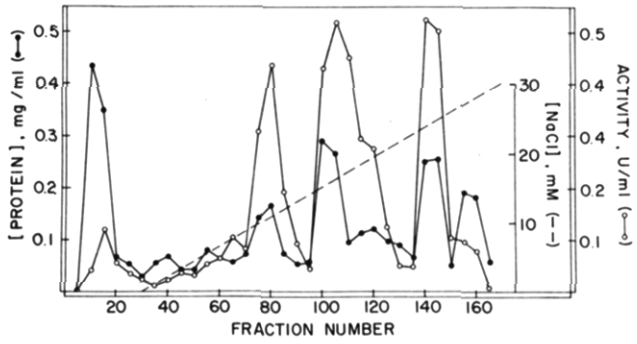

FIGURE 1: Chromatography of class I ADH isoenzymes on CM-cellulose. Approximately 217 units of unresolved class I isoenzymes equilibrated in 34 mL of 5 mM Tris–phosphate–1 mM NAD^+, pH 7.7, were applied to a 2.5 × 40 cm column of CM-cellulose equilibrated in the same buffer at 4 °C. The column was eluted with 25–300 mL of the same buffer and then with a linear 1500-mL gradient of 0–30 mM NaCl in the same buffer. Fractions of 8–10 mL were collected: (●) protein: (○) ADH activity; (---) NaCl gradient. Seven fractions were pooled for rechromatography (F-1, 5–20; F-2, 50–70; F-3, 71–90; F-4, 91–110; F-5, 111–127; F-6, 135–149; F-7, 150–165).

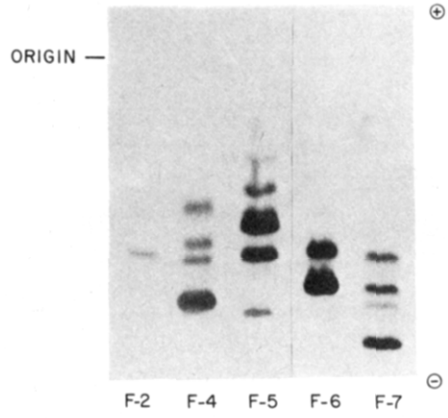

FIGURE 2: Starch gel electrophoretic patterns of pooled fractions from CM-cellulose chromatography (Figure 1).

enzymes with a Cary 219 spectrophotometer, after the sample had been dialyzed against five changes of at least 500 volumes of 10 mM Tris-HCl, pH 7.3, to remove NAD^+. Concentrations of ADH solutions were calculated by using 0.6 mL mg^{-1} cm^{-1} as the absorptivity coefficient (Lange & Vallee, 1976).

Results

Purification of Class I Isoenzymes. As a group, the class I isoenzymes of ADH that elute from CapGapp–Sepharose with 0.5 M ethanol comprise the pyrazole-sensitive ADH isoenzymes of liver homogenates (Lange & Vallee, 1976) and are devoid of both π-ADH (class II) (Li et al., 1977) and χ-ADH (class III) isoenzymes (Pares & Vallee, 1981). They are partially resolved by chromatography on CM-cellulose into multiple components (usually six to seven fractions) depending on the particular liver used (Figure 1). All ADH activity elutes from the column between 0 and 30 mM NaCl. Pooled fractions are still somewhat heterogeneous, as judged by electrophoresis on starch gels and subsequent staining for enzymatic activity (Figure 2). About 75% of the total activity applied to the column is recovered.

To isolate individual isoenzymes, each of the pooled fractions is rechromatographed on CM-cellulose at pH 7.2 (Figure 3). Fractions are pooled again based on the elution of protein and enzyme activity and assessed for homogeneity. As shown in Figure 4A, all of the fractions now contain a single, predominant enzymatic species that can be identified by comparing its electrophoretic mobility with those of the class I isoenzymes

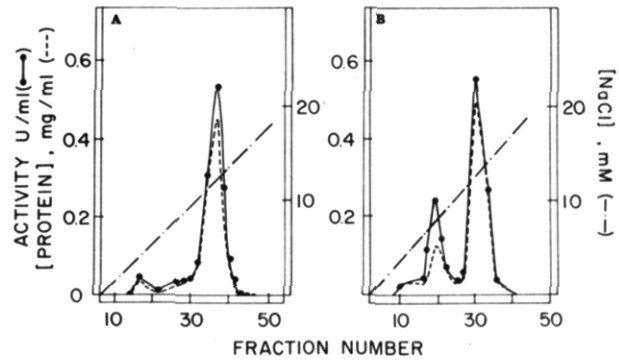

FIGURE 3: Rechromatography of class I isoenzymes of ADH on CM-cellulose. The ion-exchange resin was prepared and equilibrated and eluted as described under Experimental Procedures: (●) ADH activity; (---) protein. (A) F-4 from Figure 1: fractions 30–42 were pooled. (B) F-6 from Figure 1: fractions 18–25 and 28–35 were pooled.

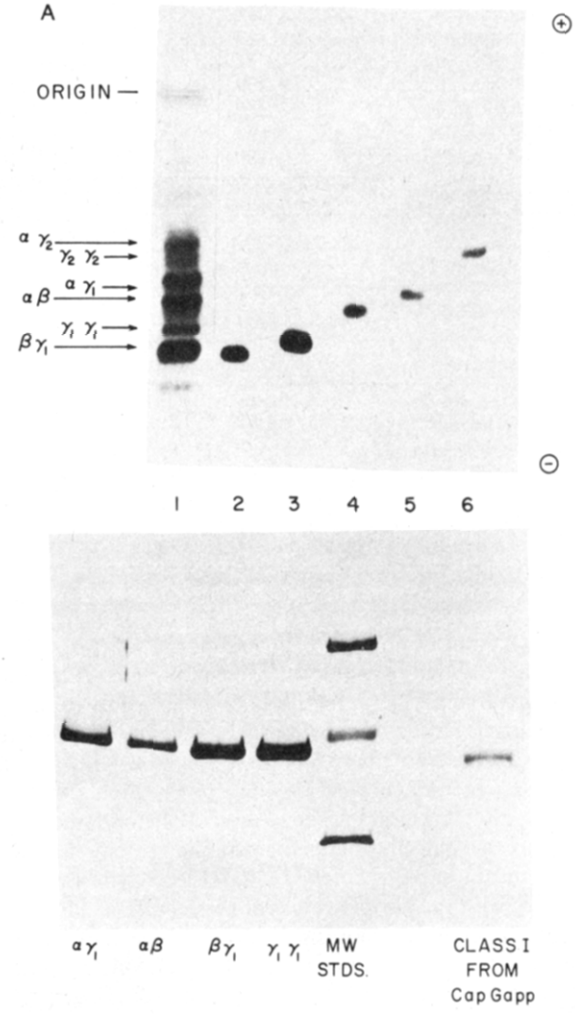

FIGURE 4: Electrophoresis of pooled peaks from rechromatography of class I isoenzymes (Figure 3). (A) Starch gel electrophoresis: lane 1, unresolved class I isoenzymes; lane 2, $\beta\gamma_1$ isolated from F-1 (Figure 1); lane 3, $\gamma_1\gamma_1$ isolated from F-6 (Figure 1); lane 4, $\alpha\beta$ isolated from lane 5, $\alpha\gamma_1$ isolated from F-4 (Figure 1); lane 6, $\gamma_2\gamma_2$ isolated from F-5 (Figure 1). (B) $NaDodSO_4$-gel electrophoresis of purified isoenzymes, a standard mixture composed of bovine serum albumin (M_r 66000), ovalbumin (M_r 46000), and carbonic anhydrase (M_r 29000), and a mixture of class I isoenzymes eluted from the CapGapp affinity resin. Gels were stained with Coomassie Blue.

in liver homogenates. Single bands reflecting $\alpha\gamma_2$-, $\gamma_2\gamma_2$-, $\alpha\gamma_1$-, $\alpha\beta_1$-, $\beta_1\gamma_2$-, $\gamma_1\gamma_1$-, $\beta_1\gamma_1$-, and $\beta_1\beta_1$-ADH isoenzymes were obtained from different livers in the course of these studies. The specific activities of all isoenzymes toward ethanol varied

Table I: K_m Values (μM) for ADH Isoenzymes toward Various Alcohols

	isoenzyme							
substrate	$\alpha\gamma_1$ (1)	$\alpha\gamma_2$ (2)	$\alpha\beta_1$ (3)	$\gamma_2\gamma_2$ (4)	$\beta_1\gamma_2$ (5)	$\gamma_1\gamma_1$ (6)	$\beta_1\gamma_1$ (7)	$\beta_1\beta_1$ (8)
(a) ethanol	1 700	1600	2 000	1 000	1 400	2 000	1 100	1 200
(b) methanol	150 000	*a*	15 000	30 000	18 000	74 000	21 000	6 000
(c) ethylene glycol	50 000	*a*	47 000	32 000	36 000	57 000	220 000	13 000
(d) benzyl alcohol	12	4	4	8	11	7	26	120
(e) octanol	8	*a*	17	*a*	5	*a*	9	*a*
(f) 16-HHA	10	*a*	9	*a*	7	26	13	12
(g) cyclohexanol	8	6	5	24	150	8	41	23 000

[a] Not determined.

Table II: k_{cat} Values (min^{-1}) for ADH Isoenzymes toward Various Alcohols

	isoenzyme							
substrate	$\alpha\gamma_1$ (1)	$\alpha\gamma_2$ (2)	$\alpha\beta_1$ (3)	$\gamma_2\gamma_2$ (4)	$\beta_1\gamma_2$ (5)	$\gamma_1\gamma_1$ (6)	$\beta_1\gamma_1$ (7)	$\beta_1\beta_1$ (8)
(a) ethanol	240	142	200	80	120	230	150	10
(b) methanol	12	*a*	12	14	12	22	12	8
(c) ethylene glycol	72	*a*	50	66	55	85	50	7
(d) benzyl alcohol	280	122	170	50	58	127	115	11
(e) octanol	260	*a*	170	*a*	70	*a*	52	*a*
(f) 16-HHA	120	*a*	100	*a*	62	208	72	10
(g) cyclohexanol	180	118	140	30	62	88	41	8

[a] Not determined.

Table III: Human Class I ADH Isoenzymes: k_{cat}/K_m Values toward Various Alcohols

	$k_{cat}/K_m \times 10^{-5}$ (M^{-1} min^{-1})							
substrate	$\alpha\gamma_1$ (1)	$\alpha\gamma_2$ (2)	$\alpha\beta_1$ (3)	$\gamma_2\gamma_2$ (4)	$\beta_1\gamma_2$ (5)	$\gamma_1\gamma_1$ (6)	$\beta_1\gamma_1$ (7)	$\beta_1\beta_1$ (8)
(a) ethanol	1.4	0.9	1.0	0.8	0.9	1.2	1.4	0.08
(b) methanol	0.0008	*a*	0.008	0.005	0.007	0.003	0.006	0.013
(c) ethylene glycol	0.014	*a*	0.010	0.020	0.015	0.015	0.002	0.054
(d) benzyl alcohol	230	300	420	63	53	180	44	0.90
(e) octanol	320	*a*	100	*a*	140	*a*	58	*a*
(f) 16-HHA	120	*a*	110	*a*	90	80	55	8.3
(g) cyclohexanol	230	200	280	13	4.1	110	18	0.003

[a] Not determined.

from 1 to 2.5 units/mg of protein, except that of $\beta_1\beta_1$-ADH, whose specific activity was 0.15 unit/mg of protein. A number of polyacrylamide gel electrophoresis procedures using nondenaturing buffer systems were employed to detect heterogeneity arising from non-ADH proteins. None of them was entirely satisfactory, however, owing to the high (>10) p*I* values of all the class I isoenzymes. Polyacrylamide gel electrophoresis buffer systems for cationic proteins were examined, but the stability of class I ADH isoenzymes was not compatible with the low pH values of these buffers. However, $NaDodSO_4$ gel electrophoresis resolved only a single species of protein for the purified isoenzymes, each of which migrated with an apparent molecular weight of 40 000 (Figure 4B).

Oxidation of Alcohols by Purified Class I ADH Isoenzymes. Due to the very broad specificity of unresolved class I ADH isoenzymes (Lange et al., 1976; Frey & Vallee, 1979; Pietruszko, 1979) it was not practical to measure kinetic parameters for the purified isoenzymes on all of the many possible alcohol substrates. Consequently, the seven alcohols selected for this study, representing primary, aromatic, and cyclic alcohols or alcohols of metabolic or toxicological significance, were readily oxidized by all the purified isoenzymes.

Values of K_m, k_{cat}, and k_{cat}/K_m, shown in Tables I, II, and III, respectively, were measured on single preparations from two or three different livers. In successive preparations the values of K_m and k_{cat} for a given isoenzyme were found to vary by no more than 3-fold when compared with those shown in Tables I and II. For different isoenzymes values of K_m vary 40 000-fold, e.g., from 4 μM for the $\alpha\gamma_2$ and $\alpha\beta_1$ isoenzymes with benzyl alcohol to 0.15 M for $\alpha\gamma_1$ -ADH acting on methanol. However, for any given alcohol—except cyclohexanol—K_m values vary by no more than 30-fold. In all instances those for ethylene glycol or methanol are always greater than those for ethanol. For the remainder of the alcohols the Michaelis constants are μM again with the exception of cyclohexanol where the K_m for $\beta_1\beta_1$-ADH is higher than that for any other alcohol examined.

The values of k_{cat} for alcohol oxidation by ADH isoenzymes vary only 40-fold overall, i.e., from 7 min^{-1} to 280 min^{-1} (Table II). Notably, k_{cat} values for methanol oxidation by any isoenzyme (row b) or those for the oxidation of any alcohol by $\beta_1\beta_1$-ADH (column 8) are almost constant. Thus, $\beta_1\beta_1$-ADH oxidizes methanol as effectively as it does any other alcohol, but it is less effective than any other class I isoenzyme toward the remainder of the alcohols.

Values of k_{cat}/K_m best reflect the specificities of the purified ADH isoenzymes (Table III). Isoenzymes containing an α subunit generally exhibit a high k_{cat}/K_m toward all substrates examined; values for $\alpha\gamma_1$, $\alpha\gamma_2$, and $\alpha\beta_1$ toward benzyl alcohol and cyclohexanol are the highest. 16-HHA is a good substrate for all isoenzymes examined (Table III); $\alpha\gamma_1$-ADH is most and $\beta_1\beta_1$-ADH is least effective in catalyzing its oxidation.

In fact, the k_{cat}/K_m values for $\beta_1\beta_1$-ADH toward all alcohols—except methanol and ethylene glycol—are lower than those for any other isoenzyme (Table III).

Of particular interest are the kinetic parameters for two sets of heterodimers $\beta_1\gamma_2$ and $\beta_1\gamma_1$ (columns 5 and 7) and those for their parent homodimers $\gamma_2\gamma_2$, $\gamma_1\gamma_1$, and $\beta_1\beta_1$ (columns 4,6, and 8) in Tables I, II, and III. The value of a given kinetic parameter obtained for a heterodimer when oxidizing a number of substrates is not the mean of those observed for the parent homodimers when acting on the same substrate. This is apparent from the K_m for cyclohexanol (Table I, line g), the k_{cat} of $\beta_1\gamma_1$ for benzyl alcohol (Table II), and the k_{cat}/K_m of $\beta_1\gamma_2$ for that substrate (Table III, line d).

Discussion

The number and diversity of isoenzymes of human liver alcohol dehydrogenases have turned out to be greater than anticipated when first recognized (Blair & Vallee, 1966). Homogenates of human liver are now known to contain multiple class I ADH isoenzymes (Smith et al., 1973; Lutsdorf et al., 1970; Li & Magnes, 1975 and references cited therein). Both the β and γ subunits show genetic polymorphism (Li & Magnes, 1975; Smith et al., 1973) and liver homogenates may contain isoenzymes composed of either γ_1 and/or γ_2 and β_1 and/or β_2 subunits. In livers having both γ_1 and γ_2 as many as 15 class I ADH isoenzymes ($\alpha\alpha$, $\alpha\gamma_1$, $\gamma_1\gamma_1$, $\gamma_1\gamma_2$, $\alpha\beta_1$, $\alpha\gamma_2$, $\beta_2\gamma_1$, $\beta_2\gamma_2$, $\beta_1\beta_2$, $\beta_2\beta_2$, $\beta_1\gamma_1$, $\beta_1\gamma_2$, $\gamma_2\gamma_2$, $\alpha\beta_2$, and $\beta_1\beta_1$) can be found. The initial procedures for isolating human ADH (Blair & Vallee, 1966; Lutstorf et al., 1970) were clearly suboptimal for the definitive recognition and purification of all of these isoenzymes. The subsequent differentiation and resolution of the isoenzymes into three classes were based on the development of CapGapp–Sepharose, an affinity resin that specifically adsorbs the class I ADH isoenzymes (Lange & Vallee, 1976; Strydom & Vallee, 1982). The approach to purifying the ADH isoenzymes used in this study is based entirely on the biospecificity of CapGapp–Sepharose.

The efficacy of affinity chromatography is illustrated in Figure 4. The material eluted from the resin is free of extraneous protein as judged by $NaDodSO_4$ and electrophoresis (Lange et al., 1976; Strydom & Vallee, 1982) and exhibits a single band with an apparent molecular weight of 40 000 (Figure 4B). Starch gel electrophoresis (Figure 2) resolves the various isoenzymes, and hence activity staining of starch gels currently provides the most effective and sensitive criterion for the assessment of enzymatic homogeneity and for the identification of these isoenzymes.

Subsequent chromatography and rechromatography on CM-cellulose provided highly purified preparations of ADH isoenzymes of the following subunit composition: $\alpha\gamma_2$, $\gamma_2\gamma_2$, $\alpha\gamma_1$, $\alpha\beta_1$, $\beta_1\gamma_2$, $\gamma_1\gamma_1$, $\beta_1\gamma_1$, and $\beta_1\beta_1$. In all livers studied $\alpha\alpha$-ADH was present in such small quantities that it could not be purified in amounts sufficient for compositional and kinetic analysis. All of the isoenzymes examined, regardless of subunit structure, are composed solely of subunits of ~40 000 daltons, consistent with the known molecular weight of ~80 000 for the undifferentiated ADH class I isoenzymes or other homogeneous mammalian ADH isoenzymes (Jörnvall, 1970). Specific activities of the purified isoenzymes toward ethanol vary from 1 to 2.5 units/mg of protein, in good agreement with preparations obtained by Lutstorf et al. (1970); $\beta_1\beta_1$-ADH, with a specific activity of 0.15 unit/mg of protein, is the notable exception.

For all these isoenzymes the optimum rates of alcohol oxidation are at alkaline pH values. Lutstorf et al. (1970) also demonstrated alkaline pH optima (between pH 9 and pH 11) for all the isoenzymes that they isolated from human liver including that initially called "atypical" (von Wartburg & Schürch, 1968) but now called ADH_{Bern}.[4] On the other hand, Yoshida et al. (1981) have purified $\beta\beta$-ADH and an "atypical" $\beta\beta$-ADH [$ADH_{Honolulu}$ using the nomenclature of Bosron et al. (1980)] and have demonstrated that while the pH optimum for ethanol oxidation of the former is between pH 10 and pH 11, that of the atypical species is at pH 8.45. Structural studies on $ADH_{Honolulu}$ (Yoshida et al., 1981) were interpreted to demonstrate that a histidyl residue is substituted for Cys-47 [corresponding to Cys-46 in horse ADH (Jörnvall, 1970)], which should be a ligand for the catalytically essential Zn atom of human ADH. Whether this structural change accounts for the lower pH optimum of $ADH_{Honolulu}$ is, however, not yet clear.[5]

The kinetic parameters reported here for individual isoenzymes are invariant for the same isoenzymes isolated from different livers. Furthermore, the K_m and k_{cat} values for ethanol are in good agreement with those measured by Bosron & Li (1982), who studied kinetic properties of $\gamma_1\gamma_1$- and $\gamma_2\gamma_2$-ADH at pH 10 and 7.5. They also communicated to us[6] that the class I ADH isoenzymes oxidized ethanol at pH 10 by classical Michaelis–Menten kinetics with k_{cat} values very similar to those presented here. However, at physiological pH values, they detected negative homotropic cooperativity for $\gamma_1\gamma_1$- and $\gamma_2\gamma_2$-ADH.

The variations in values of k_{cat} among the isoenzymes examined are relatively small (maximally 40-fold). The values for $\beta_1\beta_1$-ADH (Table II, column 8) are virtually the same for all substrates and are appreciably lower than those for any other isoenzyme toward all alcohols except methanol. In fact in all cases, the lowest values of k_{cat} are for the oxidation of methanol. These results are in good agreement with those summarized by Pietruszko (1979) for ADH isoenzymes from horse liver, rat liver, and partially purified preparations of class I human liver isoenzymes. Thus, when isoenzymes of a single species are assayed under similar conditions, values of k_{cat} are relatively invariant for primary aliphatic alcohols (from two to eight carbons in chain length) and are markedly lower for methanol. For horse liver ADH it has been established that the rate of oxidation of ethanol is limited by dissociation of NADH from the ADH·NADH complex while that of methanol is limited by the catalytic step (Brooks & Shore, 1971). Consequently, it would appear that the constancy of the values of k_{cat} for ADH isoenzymes from human liver toward primary aliphatic alcohols (C-2 and longer) reflects the rate-limiting dissociation of NADH and that of methanol reflects the catalytic step. Since this rationale should be generally applicable, the results obtained by using $\beta_1\beta_1$-ADH would seem to reflect a mechanism in which either the catalytic step is rate limiting for all substrates or the oxidiation of methanol is now limited by the dissociation rate of NADH. Whatever the basis, the $\beta_1\beta_1$ homodimer differs from the other isoenzymes by its low and relatively constant k_{cat} values, leading to the inference that this isoenzyme might serve metabolic objectives differently from those of other class I isoenzymes. For other isoenzymes, e.g., $\beta_1\gamma_1$ acting on benzyl alcohol, the values of k_{cat} may reflect a composite of rate constants representing more than one mechanistic step. Alternatively, the value of the rate constants

[4] The functional, conformational, and structural dissimilarities and/or identities of ADH_{Bern} and $ADH_{Honolulu}$ remain to be established and may or may not resolve these apparently conflicting reports.

[5] The kinetic behavior of $ADH_{Indianapolis}$, ADH_{Bern}, and $ADH_{Honolulu}$ were not examined but will be of great interest for comparison.

[6] Personal communications.

Table IV: Ratio of Observed to Calculated[a] Kinetic Parameters: $\beta_1\gamma_1$ and $\beta_1\gamma_2$

alcohol	k_{cat}		K_m		k_{cat}/K_m	
	$\beta_1\gamma_1$	$\beta_1\gamma_2$	$\beta_1\gamma_1$	$\beta_1\gamma_2$	$\beta_1\gamma_1$	$\beta_1\gamma_2$
ethanol	1.3	2.7	0.7	1.27	2.1	2.0
methanol	0.8	1.1	0.5	1.0	0.8	0.9
ethylene glycol	1.1	1.9	6.3	1.6	0.06	0.4
benzyl alcohol	1.7	1.9	0.4	0.2	0.5	1.5
octanol	1.7	1.9	0.4	0.17	0.5	1.6
16-HHA	0.7	*b*	0.7	*b*	1.0	*b*
cyclohexanol	0.9	3.3	0.004	0.01	0.3	0.63

[a] calculated = (homodimer 1 + homodimer 2)/2. [b] Not determined.

of the catalytic step may vary with the identity of the alcohol and the isoenzyme.

While the values of k_{cat} do not vary greatly with different substrates, those of K_m vary from 4 μM to 150 mM, indicating that the specificity of class I ADH isoenzymes manifests principally in substrate binding. The values of K_m and k_{cat}/K_m reveal a number of additional features related to specificity. None of the individual ADH isoenzymes of class I is uniquely reactive toward one substrate. Specificities of individual isoenzymes vary considerably, but for all of them short-chain alcohols are poorer substrates than those with longer chains or bulky R groups (rows a, b, and c, Tables I and III). This conclusion is the same as that reached in specificity studies performed with ADH isoenzymes from horse liver (Pietrusko, 1979 and references cited therein) and with those from mouse liver (Holmes et al., 1981). In these studies, K_m values for ethanol varied from 0.13 to 0.75 unit and decreased sharply as the chain length of the alcohol increased or contained an aromatic or cyclic substituent.

It is important to note that ethanol turns out to be one of the poorer substrates for human class I ADH isoenzymes, as judged by the catalytic rate constants. In addition it does not saturate χ-ADH (class III) (Pares & Vallee, 1981) and is oxidized by π-ADH with a K_m even higher than those for class I isoenzymes [33 mM (Bosron et al., 1979a)]. Thus, it appears that none of the human ADH isoenzymes characterized thus far prefers ethanol to other alcohols as a substrate.

The experimental values of the kinetic parameters for heterodimers deviate from their values as calculated from the average of their parent homodimers, i.e., by obtaining the average k_{cat}, K_m, and k_{cat}/K_m of the parent homodimers, $\beta_1\beta_1$ plus $\gamma_1\gamma_1$ and $\beta_1\beta_1$ plus $\gamma_2\gamma_2$. These would be expected to equal the experimental data obtained for $\beta_1\gamma_1$ and $\beta_1\gamma_2$ if there is no interaction of the subunits. Table IV lists the ratios of the experimental to the calculated values of the above constants for all substrates examined. A ratio of 1 would indicate that the kinetics of the heterodimers are simply the composite of the constituent homodimers; hence, there would be no interaction of the subunits in that case. However, in virtually all instances, save methanol (line b), the ratios are either greater or less than 1. Thus, the values of k_{cat}, K_m and k_{cat}/K_m of the heterodimers $\beta_1\gamma_1$ and $\beta_1\gamma_2$ deviate significantly from those anticipated if they were the average of the parent homodimers $\beta_1\beta_1$ plus $\gamma_1\gamma_1$ or plus $\gamma_2\gamma_2$, respectively.

Until now individual subunits of ADH isoenzymes from any species have not been reported to exhibit enzymatic activity. Hence, the above findings are best interpreted to reflect subunit interaction between the monomers involved, thereby becoming determinants of the kinetic parameters (Table IV). This phenomenon, well known in other instances, constitutes an effective metabolic control mechanism. Theoretically it would allow for the formation of 15 dimeric isoenzymes from 5 monomeric proteins of ADH which could have distinctive metabolic functions and missions.

The analogous isoenzymes of horse liver (EE, ES, and SS) have neither been characterized nor compared kinetically nor, in fact, have they been examined similarly for the same substrates. However, data are available on the specific activities of the ES isoenzyme for these substrates; these suggest that they are the mean of those for the EE and SS isoenzymes (Pietruszko & Theorell, 1969). Further, in studies using different substrates and inhibitions, 5β-dihydrotestosterone failed to inhibit alcohol oxidation or aldehyde reduction, and butanol did not inhibit steroid oxidation. It was concluded that in the horse enzyme heterodimer ES, the steroid-specific subunits acts independently of the ethanol-specific subunit, E (Pietruszko, 1975).

The present data show that the broad substrate specificity, previously observed for the unresolved class I ADH isoenzymes, is encountered also in each of the constituent isoenzymes of class I ADH. Clearly, it is not the sum of individually unique and markedly different substrate specificities. None of the isoenzymes of class I oxidizes ethanol in preference to the other alcohols studied. Moreover, kinetically ethanol is a poor substrate for all class I isoenzymes which constitute the predominant forms of human alcohol dehydrogenases. Thus, while physiological metabolites for class I isoenzymes have not yet been recognized, all these class I isoenzymes are very effective in oxidizing alcohols which vary greatly in structure.

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Registry No. ADH, 9031-72-5; ethanol, 64-17-5; methanol, 67-56-1; ethylene glycol, 107-21-1; benzyl alcohol, 100-51-6; octanol, 111-87-5; 16-HHA, 506-13-8; cyclohexanol, 108-93-0.

References

Bio-Rad Technical Bulletin (1978) No. 78-0791, Bio-Rad Laboratories, Richmond, CA.

Blair, A. H., & Vallee, B. L. (1966) *Biochemistry* 5, 2026–2034.

Bosron, W. F., & Li, T.-K. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 921.

Bosron, W. F., Li, T.-K., Dafeldecker, W. P. & Vallee, B. L. (1979a) *Biochemistry 18*, 1101–1105.

Bosron, W. F., Li, T.-K., & Vallee, B. L. (1979b) *Biochem. Biophys. Res. Commun. 91*, 1549–1555.

Bosron, W. F., Li, T.-K., & Vallee, B. L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5784–5788.

Brooks, R. L., & Shore, J. D. (1971) *Biochemistry, 10*, 3855–3858.

Burger, A. R., & Vallee, B. L. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1886.

Frey, W. A., & Vallee, B. L. (1979) *Biochem. Biophys. Res. Commun. 91*, 1543–1548.

Frey, W. A., & Vallee, B. L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 924–927.

Holmes, R. S., & Albanese, F. D., Whitehead, F. D., & Dudley, J. A. (1981) *J. Exp. Zool. 217*, 151–157.

Jörnvall, H. (1970) *Eur. J. Biochem. 16*, 25–40.

Lange, L. G., & Vallee, B. L. (1976) *Biochemistry 15*, 4681–4686.

Lange, L. G., Sytkowski, A. J., & Vallee, B. L. (1976) *Biochemistry 15*, 4687–4693.

Li, T.-K., & Theorell, H. (1969) *Acta Chem. Scand. 23*, 892–902.

Li, T.-K., & Magnes, L. J. (1975) *Biochem. Biophys. Res. Commun. 63*, 202–208.
Li, T.-K., Bosron, W. F., Dafeldecker, W. P., Lange, L. G., & Vallee, B. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4378–4381.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lutstorf, U. U., Schürch, P. M., & von Wartburg, J.-P. (1970) *Eur. J. Biochem. 17*, 497–508.
Pares, X., & Vallee, B. L. (1981) *Biochem. Biophys. Res. Commun. 98*, 121–130.
Pietruszko, R. (1975) in *Isozymes* (Markert, C. L., Ed.) Vol. 1, pp 707–724, Academic Press, New York.
Pietruszko, R. (1979) in *Biochemistry and Pharmacology of Ethanol* (Majchrowicz, E., & Noble, E. P., Eds.) Vol. 1, pp 87–106, Plenum Press, New York.
Pietruszko, R., & Theorell, H. (1969) *Arch. Biochem. Biophys. 131*, 288–298.
Smith, M., Hopkinson, D. A., & Harris, H. (1973) *Ann. Hum. Genet. 37*, 49–67.
Strydom, D. J., & Vallee, B. L. (1982) *Anal. Biochem. 123*, 422–429.
Vallee, B. L., & Bazzone, T. J. (1983) in *Isoenzymes: Current Topics in Biological and Medical Research* (Scandalios, J. G., Rattazzi, M. C., & Whitt, G. S., Eds.) Vol. 8 (in press).
von Wartburg, J.-P., & Schürch, P. M. (1968) *Ann N.Y. Acad. Sci. 151*, 936–946.
Yoshida, A., Impraim, C. C., & Huang, I.-Y. (1981) *J. Biol. Chem. 256*, 12430–12436.

Self-Association of the Cardiac Fatty Acid Binding Protein. Influence on Membrane-Bound, Fatty Acid Dependent Enzymes†

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ABSTRACT: The present study on the fatty acid binding protein, purified from pig heart and studied by three independent techniques (electron spin resonance, circular dichroism, and polyacrylamide gel electrophoresis), suggests that the protein self-aggregates and exists in at least four distinct molecular species. This plurality is demonstrated by the presence of four bands after electrophoretic migration at pH 7.2 and by three transitions of molar ellipticity θ_{225} that depend on protein concentration. A mathematical model is formulated to simulate the three transitions and to calculate the concentrations of the four species. The multistates manifest themselves in a complex binding capacity for fatty acid, with two sigmoidal components in the binding curve. A general equation for the curve is formulated, and the characteristic constants are evaluated by a nonlinear least-squares fit. The experimental results and their interpretation in quantitative terms lead to a theoretical evaluation of the importance of this new property of self-aggregation of the protein on the activity of membrane-bound model enzymes which are fatty acid or acyl coenzyme A dependent.

The fatty acid binding protein, of molecular weight 12 000 (Fournier et al., 1978) and cytoplasmic origin (Capron et al., 1979; Rüstow et al., 1979), discovered some 10 years ago in most organs (Levi et al., 1969; Ockner et al., 1972), is capable of binding fatty acids, acyl coenzyme A (acyl-CoA),[1] and acylcarnitine derivatives (Mishkin & Turcotte, 1974a). Of the properties studied so far, the most remarkable is the indirect control the protein exercises on the activity of important membrane enzymes such as mitochondrial ATP/ADP translocase (Barbour & Chan, 1979), mitochondrial and microsomal acyl-CoA synthetase (Wu-Rideout et al., 1976; Ockner & Manning, 1976), or the microsomal enzymes responsible for the synthesis of phospholipids and triglycerides (O'Doherty & Kuksis, 1975; Mishkin & Turcotte, 1974b; Iritani et al., 1980; Mishkin & Roncari, 1976). This protein warrants great attention as it is the only transporter described to date which is presumably capable of translocating fatty acids and their CoA derivatives in the cytoplasm. Despite the importance of the protein and its activities, no information on the driving force or the molecular mechanism of fatty acid binding has appeared. Furthermore, what data are available on the binding isotherm are not quantitatively interpretable because of the use of an impure protein preparation (Mishkin & Turcotte, 1974a) or the lack of detailed elaboration (Ketterer et al., 1976). This is apparently due to the inapplicability of the classical technique of equilibrium dialysis for long-chain fatty acids, a consequence of the restricted diffusibility of the latter.

We report here a new property for this protein; experimental evidence obtained by three independent techniques (CD, ESR, and polyacrylamide gel electrophoresis) suggests that the fatty acid binding protein purified from cardiac muscle can self-aggregate and exist in at least four molecular states. The predictable importance of this property on the transfer of fatty acid or acyl-CoA between the cytoplasm and membranes is analzyed.

Materials and Methods

Chemicals. The spin-label 12-doxylstearic acid was purchased from Syva (Palo Alto, CA) and used as the potassium salt. Acrylamide was from Serva and Pharmalyte, pH 4–6.5, from Pharmacia.

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[1] Abbreviations: ESR, electron spin resonance; CD, circular dichroism; 12-doxylstearic acid, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxyoxazolidine; acyl-CoA, acyl coenzyme A; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Temed, *N,N,N',N'*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)-aminomethane.

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