

## Dissociation and Rate Constants of Some Human Liver Alcohol Dehydrogenase Isoenzymes

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ADH from human liver forms binary complexes with NADH, associated with a blue shift of the peak of the fluorescence emission of NADH. The wavelength shift is the same for all isoenzymes but the accompanying intensification of the fluorescence is different. The fluorescence is further increased by the formation of the very tight ternary enzyme-NADH-isobutyramide complexes. These properties are similar to those for the horse liver ADH, as well as the molecular weight of  $E=40\,000$  \*\* per active site of the dimer molecule ( $EE$ ). "Stopped-flow" determined velocity constants ( $ER \rightleftharpoons E+R$ ) were found to be in good agreement with ethanol activity constants previously determined by activity measurement, confirming the validity of the ordered ternary complex mechanism also for the human ADH. No single isoenzyme activity as high as that reported by Mourad and Woronick or Drum has been found.

In 1967 Mourad and Woronick<sup>1</sup> described the isolation and crystallization of a human liver alcohol dehydrogenase (ADH) consisting of a single isoenzyme component and having a specific activity with ethanol equivalent to 60–70 % of that of the horse liver enzyme. Drum<sup>2</sup> has reported a human liver ADH isoenzyme with an activity of 120 % of the horse liver enzyme. The specific activity of the human ADH isolated by other investigators<sup>3–6</sup> was never so high. In most cases it was about half or less of the activity reported by Mourad and Woronick. These discrepancies may be attributed to human liver ADH isoenzymes with

widely varying activities. However, after the isolation of six human ADH isoenzymes Pietruszko *et al.*<sup>6</sup> were not able to find any isoenzyme whose activity was higher than half of that reported by Mourad and Woronick, and isoenzymes with ethanol activity even lower than that were found. For three of these isoenzymes we have determined the rate constants for the binary complexes of the enzyme and NADH. The dissociation constants for the reduced coenzyme ( $E+R \rightleftharpoons ER$ ) were determined for all six isoenzymes as well as dissociation constants for the ternary complexes with isobutyramide ( $EI+R \rightleftharpoons EIIR$ ). In the previous paper<sup>6</sup> it was shown that one isoenzyme (IA) was a hybrid. By a "best fit" procedure it was possible to determine the different coenzyme dissociation constants for the ternary complexes of the monomers ( $K_{EI,R}$ ). The corresponding rate constants for this isoenzyme ( $k_{EI,R\text{ on}}$  and  $k_{EI,R\text{ off}}$ ) have been determined from a similar analysis of "stopped-flow" curves.

### MATERIALS AND METHODS

Chemicals and enzyme preparations were the same as in the previous paper.<sup>6</sup> Separated isoenzymes of human ADH were stored at 0 °C in 10 % saturated ammonium sulfate solutions at pH 7.5.

*Enzyme assays* were done in 1 cm cuvettes at 340 nm at 23.5 °C in 3 ml total volume using  $NAD^+$ , 0.45 mM; ethanol, 8.6 mM, in glycine buffer, 0.062 M, pH 10.0, using a Beckman DU spectrophotometer. Glycine (Sørensen) was obtained from Merck AG, Darmstadt, Germany; isobutyramide from Eastman Organic Chemicals.

*Determination of fluorescence curves and dissociation constants.* The recording spectro-

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\*\* Abbreviations used: E, Alcohol dehydrogenase (ADH) monomer; R, NADH; I, Isobutyramide.

fluorimeter has previously been described.<sup>7</sup> The excitation wavelength was 330 nm. The fluorescence intensity was recorded at 410 or 430 nm. The fluorimeter was not compensated for the different intensity of the light source at different wavelengths. The dissociation constants were determined, as earlier described, by titrating solutions of human ADH isoenzymes with suitable increments of NADH and recording the fluorescence increase at 23.5 °C in phosphate buffer  $\mu=0.1$ , pH 7.0 in the presence or absence of 0.1 M isobutyramide. All solutions were made in water freshly distilled from alkaline permanganate.

Commercial NADH (Grade III), obtained from Sigma (U.S.A.) was used without further purification. Solutions of NADH were standardized by using acetaldehyde and yeast ADH as described previously.<sup>8</sup> The concentration of NADH was determined spectrophotometrically at 340 nm before and after oxidation to NAD by acetaldehyde + yeast ADH. The purity of NADH as judged by the amount of non-oxidizable 340 nm absorption was 96 %. NADH solutions were prepared immediately before use and used only one day.

All dissociation constants are calculated from 2–6 experimental curves giving a mean error of  $\pm 20\%$ , which also applies to the velocity constants.

**Determination of velocity constants.** A "stopped-flow" fluorescence spectrometer,<sup>9</sup> recording the increase in fluorescence proportional to the formation of the binary complex of ADH with NADH (ER) or ternary complex of ADH with NADH and isobutyramide (EIR) was used. The formation of ER and EIR complex was followed at 23.5 °C in phosphate buffer  $\mu=0.1$ . The isobutyramide concentration was 0.1 M. The excitation wavelength was 330 nm and fluorescence was recorded at 410 nm. 3  $\mu\text{N}$  E mixed with an equal volume of 3  $\mu\text{M}$  R in all ER experiments gave 1.5  $\mu\text{N}$  concentration at  $t=0$ . In the EIR determinations the concentration at  $t=0$  was  $[E]=1.5 \mu\text{N}$ ,  $[R]=0.75 \mu\text{M}$ .

The velocity constants were determined from several points of the experimental curves.  $k_{\text{on}}$  was calculated from

$$d[\text{ER}]/dt = k_{\text{on}}[\text{E}][\text{R}] - k_{\text{off}}[\text{ER}] \quad (1)$$

explicitly solved.<sup>10</sup> The 1A isobutyramide experiments were treated according to the following section.

**Determination of velocity constants for 1A isoenzyme + isobutyramide combining with NADH.** In a previous paper<sup>12</sup> it was shown that the fluorescence increase following the coupling of NADH (R) to (i) a mixture of the two isoenzymes (EE and SS) of horse liver alcohol dehydrogenase or (ii) the hybrid (ES) gave identical fluorimetric titration curves (Fig. 1). A calculation of the titration curve of ES from known dissociation constants and

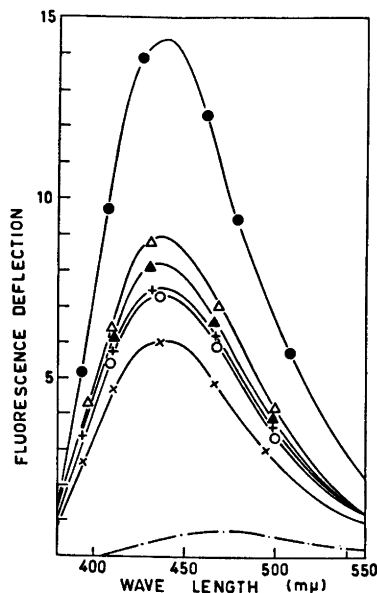


Fig. 1. Fluorescence emission spectra of 1  $\mu\text{N}$  ternary complexes of the isoenzyme with NADH and isobutyramide at the excitation wavelength of 330 nm at pH 7.0 in phosphate buffer  $\mu=0.1$ . ●, isoenzyme 1B;  $\Delta$ , isoenzyme 3B;  $\blacktriangle$ , isoenzyme 4; +, isoenzyme 2; O, isoenzyme 1A;  $\times$ , isoenzyme 3A; -.-, 1  $\mu\text{M}$  NADH. For numbering see Ref. 6.

specific fluorescences of EE and SS gave good agreement with experimental results. This means that dissociation and fluorescence of each monomer are independent of its co-monomer.

Stopped-flow experiments, mixing a solution of isoenzyme 1A in excess isobutyramide with an equal volume of a coenzyme solution gave an experimental curve quite different from the simple second order "on" and first order "off" reaction curves. Assuming different rate constants for the monomers in 1A isoenzyme ( $E_1$  and  $E_2$ ) made it possible to obtain a best fit calculated curve, Fig. 2. This is also based on the previously found dissociation constants and specific fluorescence. In these calculations different values of  $k_{\text{on},1}$  and  $k_{\text{on},2}$  were tested in order to obtain the best fit with the experimental curves.

From the equations

$$\frac{d[\text{E}_1\text{IR}]}{dt} = k_{\text{on},1}[\text{E}_1\text{I}][\text{R}] - k_{\text{off},1}[\text{E}_1\text{IR}] \quad (2)$$

$$\frac{d[\text{E}_2\text{IR}]}{dt} = k_{\text{on},2}[\text{E}_2\text{I}][\text{R}] - k_{\text{off},2}[\text{E}_2\text{IR}] \quad (3)$$

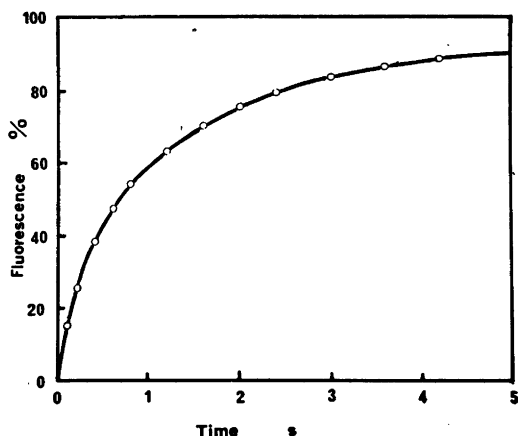


Fig. 2. Stopped-flow. Solid curve, experimental:  $0.93 \mu\text{N}$  of 1A isoenzyme mixed with  $0.75 \mu\text{M}$  NADH at  $t=0$ . Concentrations after mixture. Open Circles: calculated values for  $K_1=2 \text{ nM}$ ,  $K_2=5 \text{ nM}$ ,  $F_1=5 \text{ cm}/\mu\text{M}$ ,  $F_2=29 \text{ cm}/\mu\text{M}$ ,  $k_{\text{on}1}=8 \mu\text{M}^{-1} \text{ s}^{-1}$ ,  $k_{\text{on}2}=1.75 \mu\text{M}^{-1} \text{ s}^{-1}$ .

a total fluorescence

$$F_{\text{tot}} = F_1[E_1IR] + F_2[E_2IR] + F_R[R] \quad (4)$$

was calculated with a programmed Canola 164P desk calculator. (Specific fluorescence for  $E_1IR = F_1$ , for  $E_2IR = F_2$  and for  $R = F_R$ ).

The back reaction did not affect the calculations in the treated part of the curve due to the very high affinity between enzyme and coenzyme in isobutyramide.

The corresponding treatment of binary binding in the absence of isobutyramide was not possible due to the lower fluorescence and higher dissociation in this case. The given binary constants thus represent mean values for the two different dimer halves of the 1A hybrid isoenzyme.

Table 1. Human ADH isoenzymes: Specific fluorescence of binary complexes with NADH and ternary complexes with NADH and isobutyramide. The excitation wavelength was 330 nm.

Isoenzyme	Fluorescence increase at 410 nm "Q" values		Fluorescence increase at 430 nm fluorescence maximum	
	1 $\mu\text{M}$ ER/ 1 $\mu\text{M}$ NADH	1 $\mu\text{M}$ EIR/ 1 $\mu\text{M}$ NADH	1 $\mu\text{M}$ ER/ 1 $\mu\text{M}$ NADH	1 $\mu\text{M}$ EIR/ 1 $\mu\text{M}$ NADH
1A	6.9	40 <sup>a</sup>	3.3	18
1B	5.1	73	2.1	35
2		41		18
3A		33		15
3B	9.7	46	4.7	22
4		42		20

<sup>a</sup> Total increase: 1A is a hybrid with the two halves giving ternary increase factors 12 and 68, resp.

## RESULTS

The fluorescence peak of NADH at 470 nm is shifted to 430 nm upon binding to human ADH and the intensity is increased. Table 1 gives the increase at 430 nm and at 410 nm. Table 1 also gives the fluorescence increase for the ternary complex with isobutyramide. Fluorescence curves for the ternary complexes are given in Fig. 1 showing identical shape and position of peak but very different fluorescence intensities for all isoenzymes. The value for isoenzyme 1A is the total fluorescence increase for the two different dimer halves found in a previous paper.<sup>6</sup>

The dissociation constants of NADH with several isoenzymes of human ADH determined in the presence of excess isobutyramide are shown in Table 2. The values for different isoenzymes of human ADH vary between 0.002 and  $0.007 \mu\text{M}$ , not much different from that determined for horse liver ADH,  $0.0055 \mu\text{M}$ .<sup>11</sup>

The dissociation constants in the absence of isobutyramide are shown in Table 3. They are about one order of magnitude greater than the corresponding constants in the presence of isobutyramide and two to six times lower than the corresponding constants established for horse liver ADH.<sup>12</sup>

The "on" velocity constants for NADH with isoenzymes 1A, 1B and 3B as determined by "stopped-flow" are also listed in Table 3.  $k_{\text{off}}$  is calculated as  $k_{\text{off}} = K_{\text{ER}}k_{\text{on}}$ . The values so obtained for isoenzymes 1A, 1B and 3B are listed in Table 3 and compared with the turnover numbers at  $V_{\text{max}}$  previously obtained in the same pH, temperature and buffer. It can be seen that the  $k_{\text{off}}$  values calculated from

Table 2. Human liver ADH isoenzymes: Dissociation constants with NADH in the presence of 0.1 M isobutyramide at pH 7.0 and 23.5 °C. Rate constants for 1A and 1B isoenzymes. All values were determined in phosphate buffer  $\mu = 0.1$ , pH 7.0 containing 0.1 M isobutyramide, 23.5 °C.

Isoenzyme	1A	1B	2	3A	3B	4
$K_{EI,R}$ ( $\mu\text{M}$ )	0.002 <sup>a</sup> , 0.005 <sup>b</sup>	0.007	0.005	0.005	0.003	0.007
$k_{on,EI,R}$ ( $\mu\text{M}^{-1} \text{s}^{-1}$ )	8 <sup>a</sup> , 1.8 <sup>b</sup>	0.8				
$k_{off\ EI,R}$ ( $\text{s}^{-1}$ ) calc.	0.016 <sup>a</sup> 0.009 <sup>b</sup>	0.006				

<sup>a</sup>  $K_{EI,R}$ ,  $k_{on}$  and  $k_{off}$  for the monomer of the enzyme with the highest affinity for NADH. <sup>b</sup>  $K_{EI,R}$ ,  $k_{on}$  and  $k_{off}$  for the other monomer.

Table 3. Dissociation and rate constants for the formation and decomposition of ER complexes of NADH with different human isoenzymes as compared with previously found turnover numbers,<sup>6</sup> obtained with 500  $\mu\text{M}$  NAD<sup>+</sup> and varying concentrations of ethanol. All values were determined in phosphate buffer  $\mu = 0.1$ , pH 7.0 at 23.5 °C.

Isoenzyme	1A	1B	2	3A	3B	4
$K_{E,R}$ ( $\mu\text{M}$ )	0.06	0.13	0.09	0.05	0.06	0.03
$k_{on}$ ( $\mu\text{M}^{-1} \text{s}^{-1}$ )	9	2.5	—	—	5	—
$k_{off, calc.}$ ( $\text{s}^{-1}$ )	0.5	0.3	—	—	0.3	—
Turnover No. (Ref. 6) (act. site) <sup>-1</sup> , $\text{s}^{-1}$	0.41	0.18	—	—	0.28	—

directly determined  $k_{ER}$  and  $k_{on}$  are very close to the values of turnover numbers determined kinetically. A curve calculated from the velocity constants given in Table 2 was in good agreement with a slow relaxation curve in an earlier experiment with isobutyramide.<sup>6</sup>

## DISCUSSION

The rate limiting process in human as well as horse liver ADH ethanol oxidation is the dissociation of the ER complex. It is likely therefore that the ordered ternary complex mechanism<sup>13</sup> is also operative in the ethanol dehydrogenation by the human liver ADH at pH 7.0.

The relative low activity of the human ADH is fully explainable: The "off" velocity constant,  $k_{off}$ , in  $\text{ER} \rightleftharpoons \text{E} + \text{R}$  is  $0.3 \text{ s}^{-1}$  in human ADH, as compared with  $3 \text{ s}^{-1}$  in horse ADH.

When  $k_{on}$  values with and without isobutyramide are compared (Table 3 and 2) it can be seen that isobutyramide lowers the "on" velocities.

With the horse ADH it has been established that  $k_{on}$  values in the presence and absence of isobutyramide are the same.<sup>14</sup> The considerably lower dissociation constants between human enzyme and NADH (with isobutyramide) are mainly due to the considerably lower (50 times) "off" velocity constants overtaking the smaller decrease of "on" velocity constants.

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