Spectrophotofluorometric Determination of the Dissociation Constants of Amides from the Enzyme-Reduced Coenzyme Complex of Liver Alcohol Dehydrogenase

CHARLES L. WORONICK*,**

Nobel Medical Institute, Department of Biochemistry, Stockholm, Sweden

Spectrophotofluorometric methods for measuring the dissociation constants of binary and ternary complexes of liver alcohol dehydrogenase with DPNH *** and amides have been developed by Winer and Theorell [1]. By using DPNH to titrate mixtures of enzyme plus amide, the dissociation constants for the saturated hydrocarbon amides n-C_1 to n-C_6, as well as for isobutyramide, were determined. The present communication describes the determination of the dissociation constants $K_{ER,I}$ for a group of branched, unsaturated, and polar amides which had previously been studied as inhibitors in the liver alcohol dehydrogenase system [2]. The values for $K_{ER,I}$ were determined by direct titration of the fluorescent ER complex with amides. The $K_{ER,I}$ values obtained were found to be equal, within the limits of experimental error, to the values of the Michaelis inhibitor constants previously obtained [2].

Experimental. The preparation of liver alcohol dehydrogenase and of most of the reagents has been briefly described in a previous communication [2]. Hopkin and Williams benzamide, m.p. 128–130°, was recrystallized twice from water. A commercial grade of nicotinamide, m.p. 129–131°, was used without purification.

The titrations were performed at 23.5 ±0.1° in 0.1 μ sodium phosphate buffer pH 7.05±0.05. The reaction mixture was excited with light at 330 mμ, and the fluorescence at 410 mμ was measured. The DPNH concentration in the stock solution was determined spectrophotometrically at 340 mμ. The enzyme concentration in the stock solution was determined by titrating the enzyme-isobutyramide complex with DPNH [1]. The enzyme concentration refers to the normality of the enzyme with respect to the number of binding sites for DPNH, a 1 M solution being 2 N.

The experiments were performed as follows: To a cuvette was added buffer solution, 10 μM DPNH and 0.5–1 μN enzyme, after which the mixture, which now contained the fluorescent ER complex, was titrated with suitable aliquots of amide solution. The fluorescence was measured after each addition. The fluorescence measurements were then corrected for dilution, and for quenching of the fluorescence of the unbound DPNH, by titrating the same concentration of DPNH alone with the amide solution.

The dissociation constants were obtained graphically by plotting the reciprocal of the absolute value of the total change in fluorescence *versus* the reciprocal of the amide concentration (Fig. 1). The titrations follow the equation:

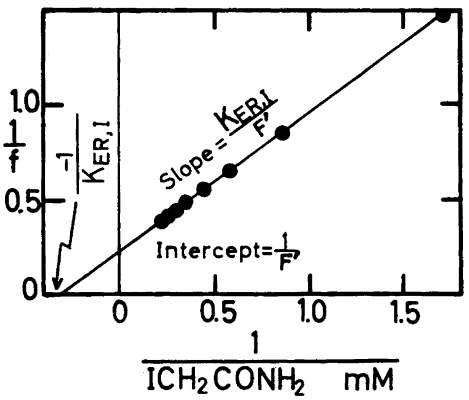

Fig. 1. Graphical method for determining the values of $K_{ER,I}$. In this case, the ER complex was titrated with 0.587 mM increments of iodoacetamide, and the value for $K_{ER,I}$ was found to be 2.6 mM. The symbols used are described in the text.

* Postdoctoral Fellow of the National Science Foundation, Washington, D.C.

** Present address: Department of Chemistry, Brown University, Providence, Rhode Island.

*** Abbreviations used: DPNH = R = Reduced diphosphopyridine nucleotide; E = Enzyme; I = Inhibitor; ER = Enzyme–DPNH binary complex; ERI = Enzyme–DPNH–Inhibitor ternary complex; Q_{ER} = Fluorescence of ER complex/Fluorescence of DPNH; Q_{ERI} = Fluorescence of ERI complex/Fluorescence of DPNH; $K_{ER,I}$ = [ER][I]/[ERI].

Table 1. Summary of results.

Amide	Q_{ERI}	$K_{ER,I}$ mM	Michaelis K_I * mM
Fluoroacetamide	3	33	31
Difluoroacetamide	0.08	79	59
Trifluroacetamide	0.4	41	40
Chloroacetamide	0.7	7.5	6.3
Dichloroacetamide	0	3.9	4.7
Trichloroacetamide	0	13	13
Bromoacetamide	0	4.1	3.9
Dibromoacetamide	0	3.0	2.7
Iodoacetamide	0	2.4	1.8
α, α-Diethylacetamide	25	0.39	0.29
Trimethylacetamide	32	3.5	2.2
Acrylamide	0	4.6	5.3
α-Methylacrylamide	0.9	0.93	0.81
Crotonamide	0.1	0.94	1.0
Tiglamide	1	0.64	0.50
Benzamide	0.3	0.47	
Nicotinamide	0	42	

* Data taken from Ref. [2].

$$\frac{1}{f} = \left[\frac{K_{ER,I}}{F'} \times \frac{1}{[I]}\right] + \frac{1}{F'}$$

where f is the absolute value of the total change in fluorescence at each inhibitor concentration, F' is the absolute value of the change in fluorescence at infinite inhibitor concentration, and [I] is the inhibitor concentration.

Results and discussion. In Table 1 the values of $K_{ER,I}$ are presented together with the Michaelis inhibitor constants obtained previously [2]. The agreement between the 2 sets of data is quite good when one considers the fact that they were obtained by entirely different experimental procedures. This confirms the interpretation [2] that the Michaelis inhibitor constant reflects competition between amide and aldehyde for the ER complex that is formed at the beginning of the catalytic cycle [3].

In almost all cases, the Q_{ERI} values are much smaller than Q_{ER} (which was found to be 12.8), and the Q_{ERI} values reported by Winer and Theorell [1]. The following general rules appear to apply under these conditions: If the amide has a pi bond (including the pi bonds of aromatic systems) in the α-position, or has an α-substituent with unshared electrons, the fluorescence of the ternary ERI complex will be much less than that of the binary ER complex. If the amide is saturated and has a short alkyl branch in the α-position, the fluorescence of the ERI complex will be much greater than that of the ER complex. If the amide is saturated, and is unbranched, or has a branch at a position other than the α-position, the fluorescence of the ERI complex will not differ much from that of the ER complex. The rules are stated in order of decreasing precedence. Thus, the ERI complex involving α-methylacrylamide is relatively non-fluorescent, whereas the complex involving isobutyramide is highly fluorescent. These results may indicate that in the ternary complex the substituent attached to the amide group is in close proximity to the reduced nicotinamide ring of DPNH.

In Fig. 2, the $K_{ER,I}$ values from this paper, as well as the values from the papers of Theorell and coworkers [1,4], are plotted according to the $\varrho^*\sigma^*$ equation of Taft [5]. The values for most of the saturated straight chain hydrocarbon amides appear to fall on one line. The monohalogenated acetamides fall on a separate curve that is displaced towards positive sigma values. The dihalogenated and trihalogenated acetamides are successively displaced in the same direction. These displacements are in the direction of greater stability

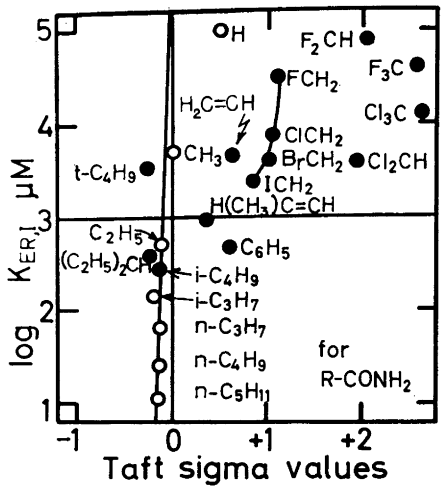

Fig. 2. The values for $K_{ER,I}$ plotted according to the Taft $\varrho^*\sigma^*$ equation. The σ^* values for the n-C_5H_{11} and CF_3 substituents were estimated as described by Taft, [5] while the σ^* value for the CH_2CH substituent was taken from Ref. [10]. The $K_{ER,I}$ values indicated by open circles were taken from Ref. [1], excepting for the value for the i-C_3H_7 substituents which was taken from Ref. [4]. The $K_{ER,I}$ value (270 μM) for the i-C_4H_9 substituent was obtained kinetically by the method described in Ref. [2].

than would be expected if the $\varrho^*\sigma^*$ equation were strictly followed. Similar results were obtained by Drago and coworkers [6,7] when they plotted the equilibrium constants of amide-iodine and amide-phenol complexes according to the Taft $\varrho^*\sigma^*$ equation.

Charton (personal communication) has suggested that a better correlation might be obtained if the values of log $K_{ER,I}$ for the amides were plotted against the Hammett sigma values for *para*-substituted benzoic acids [8]. When this was done it was found that the values for the C_2H_5, CH_3CH = CH, CH_2Cl, $CHCl_2$, CCl_3 and CF_3 substituents fell on a line that follows the equation log $K_{E,RI}$ (μM) = 2.7 σ_{para} + 3.1. The correlation coefficient was 0.99 and the standard deviation [9] was 0.26. The values for the H, CH_3, n-C_3H_7, i-C_3H_7, n-C_4H_9, t-C_4H_9 and C_6H_5 substituents deviated from this line. A more detailed report will be submitted for publication at a later date.

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Spectrophotofluorometric Determination of the Dissociation Constants of Relatively Nonfluorescent Liver Alcohol Dehydrogenase Complexes with Amides and Coenzyme

CHARLES L. WORONICK *,**

Nobel Medical Institute, Department of Biochemistry, Stockholm, Sweden

In the preceding paper, [1] the dissociation constants, $K_{ER,I}$, for a group of amides that form relatively nonfluorescent ERI

* Postdoctoral Fellow of the National Science Foundation, Washington, D.C., 1959–1961, and of the National Cancer Institute of the National Institutes of Health, Bethesda, Maryland, 1961–1962.

** Present address: Department of Chemistry, Brown University, Providence 12, Rhode Island.