UTILIZATION OF A RECYCLIZATION REACTION FOR THE ANALYSIS OF THE MECHANISM OF ENZYMIC ALCOHOL OXIDATION

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The kinetics of recyclization reactions catalyzed by horse liver alcohol dehydrogenase in the presence of a coenzyme, *p*-nitrosodimethylaniline, and various alcohols was studied from theoretical and experimental point of view. It was found that *p*-nitrosodimethylaniline is an oxidized substrate of the alcohol dehydrogenase suitable for testing basic kinetic parameters of various alcohols, especially for checking the kinetic importance of ternary enzyme–NAD–alcohol complexes.

Several recyclization methods for the determination of nicotine amide coenzymes have been described[1–3]. They are based on the cooperation of two NAD- or NADP-dependent dehydrogenases affecting the corresponding substrates. A small amount of the tested coenzyme is reduced by one and oxidized by the other enzyme. Thus the coenzyme is "recycled" in both forms. The quantity of one of the formed products (or of one of the reacting substrates) measured after a definite time of reaction is proportional to the catalytic quantity of the coenzyme present.

For the determination of NAD and NADH, recyclization reactions using only one enzyme (alcohol dehydrogenase, ADH) were also suggested[3,4]. These methods make use of the broad substrate specificity of ADH. If the enzyme is the reaction rate limiting component, the same principle can be applied for the determination of ADH activity[5,6]. The problem of "one enzyme" recyclization reactions was analyzed by authors specialized in enzyme kinetics with regard to the possibility of its use in the diagnostics of enzymic mechanisms. Fundamental contributions were published by Wratten and Cleland[7] and by Dalziel and Dickinson[8].

This paper deals with some kinetic problems connected with one enzyme recyclizations and with the practical application of a colored substrate of ADH, *p*-nitrosodimethylaniline (NDMA), for the diagnostics of the mechanism of alcohol oxidation by this enzyme. General analytical aspects of one enzyme recyclization reactions are discussed elsewhere[9].

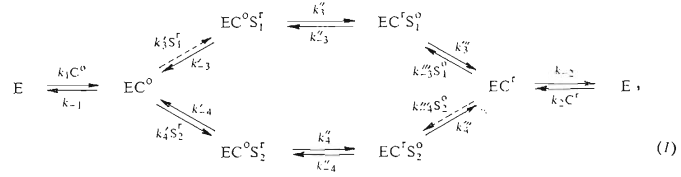
MATERIALS AND METHODS

Reagents. ADH was isolated[10] from horse liver. The enzyme concentration was determined from activity measurements[11] and is expressed in subunit molar concentrations. NAD (grade I) was from Boehringer (Mannheim), NDMA was prepared synthetically[12]. The alcohols were purified by distillation. The traces of corresponding aldehydes were removed from the furyl alcohol (International Enzymes) and the benzyl alcohol (Fluka)[13].

Spectrophotometric measurements were made in a 0·1M sodium phosphate buffer, pH 8·5, at 25°C using a Cary 118 spectrophotometer. The reaction rates were determined from the absorbance decrease at 440 nm (absorption maximum of NDMA, $\varepsilon_{440} = 35{\cdot}4\text{mM}^{-1}\,\text{cm}^{-1}$). The reactions were started by adding small amounts of the enzyme.

RESULTS AND DISCUSSION

Assuming the validity of an ordered mechanism, the recyclization reaction can be described using following scheme



(*1*)

where E is the enzyme, C^0 and C^r are two coenzyme forms (NAD and NADH, for instance), S_1^r, S_1^0 and S_2^r, S_2^0 are corresponding substrate pairs (*i.e.* alcohols S_1^r and S_2^r and aldehydes S_1^0 and S_2^0 in the case of ADH), k are rate constants. Dashed arrows in Scheme 1 stand for reaction steps not taken into account in the equation for the initial rate (the initial conditions are $S_1^r = S_2^0 = 0$). E_{tot} is the total enzyme concentration.

In the case that the Theorell–Chance mechanism is effective with respect to both substrates (*i.e.* ternary complexes in Scheme 1 are not kinetically important), a simple scheme is valid for initial conditions $S_0^r = S_2^0 = 0$

$$E \underset{k_{-1}}{\overset{k_1C^0}{\rightleftharpoons}} EC^0 \underset{k_4S_2^r}{\overset{k_{-3}S_1^0}{\rightleftharpoons}} EC^r \underset{k_2C^r}{\overset{k_{-2}}{\rightleftharpoons}} E \qquad (2)$$

Cases can also be considered in which only one of the substrates reacts according to the Theorell–Chance mechanism (a "mixed" type). Assuming that the decrease of concentration S_1^0 (or increase of S_1^r) can be measured, the initial reaction velocity can be defined as $v_0 = -(dS_1^0/dt)_{t\to 0}$ ($S_1^r = S_2^0 = 0$). The rate equations (calculated for instance according to Fromm[14]) are relatively simple for the case of saturation concentration of one coenzyme form and zero concentration of the other form (Tables I and II). In addition to the simple form, the advantage of these conditions lies in the fact that the possibility of enzyme–substrate binary complex formation is highly suppressed. Hence even the systems with random kinetics approach the ordered mechanism.

TABLE I

Interpretation of kinetic coefficients A for a one enzyme recyclization reaction (comp. Schemes 1 and 2). Initial concentrations of the coenzyme forms $C^r \to \infty$, $C^0 = 0$, initial reaction velocity $v_0 = -(dS_1^0/dt)_{t\to 0}$ (for $S_0^r = S_2^0 = 0$). Rate equation $E_{tot}/v_0 = (A_1 S_2^r + A_2)/(S_2^r + A_3) + A_4/S_1^0$

Substrates S_1^0 S_2^r	Mechanisms for substrates			
	ordered ordered	ordered Theorell–Chance	Theorell–Chance ordered	Theorell–Chance Theorell–Chance
A_1	$X_3 + X_4$	X_3	X_4	$\to 0$
A_2	$(k_{-1}X_3 + 1)\,Y_4$	$(k_{-1}X_3 + 1)\,1/k_4$	Y_4	$1/k_4$
A_3	$k_{-1}Y_4$	k_{-1}/k_4	$k_{-1}Y_4$	k_{-1}/k_4
A_4	Y_3	Y_3	$1/k_{-3}$	$1/k_{-3}$

$$X_3 = \frac{k'_{-3} + k''_{-3} + k''_3}{k'_{-3}k''_{-3}}, \quad X_4 = \frac{k''_4 + k''_{-4} + k'''_4}{k''_4 k'''_4},$$

$$Y_3 = \frac{k'_{-3}k''_{-3} + k'_{-3}k'''_3 + k''_3 k'''_3}{k'_{-3}k''_{-3}k'''_{-3}}, \quad Y_4 = \frac{k'_{-4}k''_{-4} + k'_{-4}k'''_4 + k''_4 k'''_4}{k'_4 k''_4 k'''_4}$$

TABLE II

Interpretation of kinetic coefficients B for a one enzyme recyclization reaction (comp. Schemes 1 and 2). Initial concentrations of the coenzyme forms $C^r = 0$, $C^0 \to \infty$, initial reaction velocity $v_0 = -(dS_1^0/dt)_{t\to 0}$ (for $S_1^r = S_2^0 = 0$). Rate equation $E_{tot}/v_0 = B_1 + B_2/S_1^0 + B_3/S_1^r + B_4/(S_1^0 S_2^r)$

Substrates S_1^0 S_2^r	Mechanisms for substrates			
	ordered ordered	ordered Theorell–Chance	Theorell–Chance ordered	Theorell–Chance Theorell–Chance
B_1[a]	$X_3 + X_4$	X_3	X_4	$\to 0$
B_2	$(k_{-2}X_4 + 1)\,Y_3$	Y_3	$(k_{-2}X_4 + 1)\,1/k_{-3}$	$1/k_{-3}$
B_3	Y_4	$1/k_4$	Y_4	$1/k_4$
B_4	$k_{-2}Y_3Y_4$	$(k_{-2}/k_4)\,Y_3$	$(k_{-2}/k_{-3})\,Y_4$	$k_{-2}/k_{-3}k_4$

[a] Terms B_1 and A_1 are identical (Table I), X_3, X_4, Y_3 and Y_4 are defined in Table I.

The Tables I and II summarized the rate equations for the recyclization systems with substrate branches of the ordered and Theorell–Chance type and for the mechanisms of the "mixed" type. By comparing the expressions for the mechanisms discussed, two basic rules are apparent concerning the simplification of the ordered branch to the Theorell–Chance type branch.

1) $$\frac{k'_{-3} + k''_{-3} + k''_3}{k'_{-3}k''_{-3}} \to 0 \quad \text{or} \quad \frac{k''_4 + k''_{-4} + k'''_4}{k''_4 k'''_4} \to 0\,, \tag{3}$$

which is fulfilled if k'_{-3}, $k''_{-3} \gg 1$ and $k''_3 \ll k'_{-3}k''_{-3}$ or k''_4, $k'''_4 \gg 1$ and $k''_{-4} \ll k''_4 k'''_4$ (*i.e.* if the reaction rates of ternary complexes are high enough).

2) $$\frac{k'_{-3}k''_{-3} + k'_{-3}k'''_3 + k''_3 k'''_3}{k'_{-3}k''_{-3}k'''_{-3}} = 1/k_{-3} \quad \text{or} \quad \frac{k'_{-4}k''_{-4} + k'_{-4}k'''_4 + k''_4 k'''_4}{k'_4 k''_4 k'''_4} = 1/k_4 \tag{4}$$

If the dissociation of the substrates S_1^0 and S_2^r from the corresponding ternary complexes is not too fast, *i.e.* if the values of k''_3 or k'_{-4} are not too high (and if the inequalities mentioned in the first condition are valid) the constants k_{-3} or k_4 characterizing the complex Theorell–Chance branches (compare Scheme 2) are roughly equal to the rate constants for the association of substrates with the enzyme–coenzyme complexes (*i.e.* $k_{-3} \approx k'''_{-3}$ and $k_4 \approx k'_4$). Similar criteria were formulated[15] for a general two substrate kinetics.

In the case of a strong association of coenzymes with the enzyme (k_{-1}, $k_{-2} \to 0$) rate equations given in Tables I and II are reduced to the forms typical for a ping-pong mechanism: $E_{tot}/v_0 = A_1 + A_2/S_2^r + A_4/S_1^0$ and $E_{tot}/v_0 = B_1 + B_2/S_1^0 + B_3/S_2^r$. The ping-pong kinctics can thus be considered as a special case of the mechanism discussed.

If the initial reaction velocities in the presence of one of the saturating substrates are measured, following simple equations enabling the determination of coefficients B_1, B_2 and B_3 can be written for high C^0 concentrations (compare Table II)

$$E_{tot}/v_0 \approx B_1 + B_2/S_1^0 \quad (\text{for } S_2^r \to \infty)$$
$$E_{tot}/v_0 \approx B_1 + B_3/S_2^r \quad (\text{for } S_1^0 \to \infty) \tag{5}$$

For the other coenzyme form (C^r) these equations are somewhat more complicated (compare Table I).

It is obvious that coefficients B_1 and B_3 characterize the "substrate" properties of S_2^r (*i.e.* of the alcohol in the case of ADH), while B_2 has a more complex character. Coefficient B_3 is identical with the coefficient Φ_2 in an equation put forward by Dalziel

and Dickinson[16] for the substrate characterization with respect to ADH kinetics in the presence of an alcohol and NAD. Coefficient B_1 is of fundamental importance for the consideration of the basic features of the mechanism – the more the system approaches the Theorell–Chance mechanism the lower is B_1. If at least the kinetics of the substrate S_1^0 follows the Theorell–Chance mechanism then a linear plot of B_2 against B_1 can be obtained with the slope equal to k_{-2}/k_{-3} and the intercept $1/k_{-3}$ for various substrates S_2^r.

p-Nitrosodimethylaniline (NDMA) is an oxidized substrate for ADH (S_1^0) suitable for testing the mechanism of alcohol (S_2^r) dehydrogenation for following reasons. NDMA is a chromophore substrate, which is reduced by ADH to a colorless hydroxylamine derivative undergoing a rapid elimination of the hydroxyl group and yielding benzoquinone diimine[17,18]. The products of the enzymic redox elimination reaction are no more substrates for ADH and therefore only a small risk of a reverse reaction exists (compare Scheme 1). Another important reason for the choice of NDOA is the fact that this analogue has the lowest K_m value of all known ADH substrates and a very high maximum velocity[17]. In addition, the rate of the enzymic conversion of NDMA is limited by its diffusion to the active center of ADH. Therefore the in-

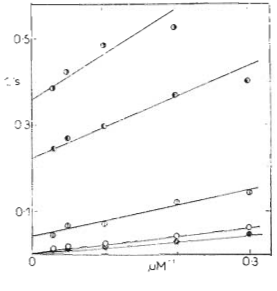


FIG. 1

Double reciprocal plots of E_{tot}/v_0 *vs* $1/[NDMA]$ for various alcohols. Concentration of substances present in the reaction mixture 0·3 mM NAD, 2 nM ADH, 1·8 mM 1-butanol (●), 5 mM ethanol (○), 35 mM 2-butanol (⊙), 6 mM benzyl alcohol (◐) and 1·8 mM furyl alcohol (◑). E_{tot} is the total ADH concentration, v_0 is the initial velocity of NDMA reduction

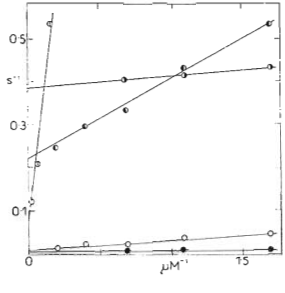


FIG. 2

Double reciprocal plots E_{tot}/v_0 *vs* $1/[alcohol]$ for various alcohols. Concentrations of the substances present in the reaction mixture 0·3 mM NAD, 2 nM ADH, 60 μM NDMA. The symbols used are the same as in Fig. 1, further conditions are described in Materials and Methods

involvement of the Theorell–Chance mechanism can be supposed. The only disadvantage of this substance is the fact[18] that at high NADH concentrations a reaction of the products formed from NDMA with NADH can occur. Therefore only the behaviour of NDMA and alcohols in the presence of NAD was analyzed.

The results of the application of equations (5) are presented in Fig. 1 and 2. Fig. 1 shows double reciprocal plots of the initial reaction velocities *vs* NDMA concentration (under conditions of saturating concentrations of NAD and the tested alcohol), corresponding dependences on reciprocal alcohol concentration (under conditions of saturating NAD and NDMA concentrations) are shown in Fig. 2. The slopes and intercepts of the straight lines in Fig. 1 substantially differ for various alcohols tested. The plot of the slopes against the intercepts from Fig. 1 gives a linear dependence, as predicted in the theoretical part for the case of S_1^0 following the Theorell–Chance mechanism. The intercept values of the dependences for individual alcohols in Fig. 1 and 2 are practically the same (coefficient B_1 in equations (5)), whereas the slopes for individual alcohols in Fig. 1 and 2 differ (coefficients B_2 and B_3 in equations (5)).

The straight line slopes in Fig.2 (B_3) characterize the substrate properties of individual alcohols and are in qualitative agreement with published values[16,19].1-Butanol is an excellent substrate for the horse liver ADH, ethanol and furyl alcohol are good substrates, whereas 2-butanol belongs to the bad substrates with a high K_m. With regard to the fact that the published data were obtained at other pH values, no quantitative comparison could be made.

The intercepts of the straight lines in Fig. 1 and 2 (corresponding to coefficients B_1 *i.e.* to the reciprocal maximum velocities) represent a relative measure of the transformation rates of ternary ADH–NAD–alcohol complexes (EC^0S_2') for various alcohols. The lower are these conversion rates the higher are the values of corresponding B_1 intercepts. The intercepts determined for primary alcohols (ethanol and 1-butanol) are practically negligible, *i.e.* the corresponding reaction rates are high provided that NAD, NDMA and alcohol are in saturating concentrations. For these alcohols the Theorell–Chance approximation assuming kinetic negligibility of ternary complexes is valid. On the other hand the intercept values for 2-butanol, benzyl alcohol and furyl alcohol are relatively high, thus for these substrates the formation of ternary complexes is kinetically important (Scheme 1 – lower branch). For the primary aliphatic alcohols the Theorell–Chance mechanism is approximately valid[16], whereas in the case of secondary and aromatic alcohols a slow transfer of the hydride anion in the ternary complex or a slow dissociation of the corresponding aldehyde from the ternary complex[7,16] were described.

The kinetic analysis of a one enzyme recyclization system involving ADH, NAD, NDMA and alcohol is more advantageous than the classical stationary diagnostics of the reaction mechanism of alcohol conversion for following reasons. It is sufficient to measure the reaction rate in one direction only (in a system of NAD,

NDMA and alcohol), whereas the conventional analysis requires data for alcohol oxidation and for the reduction of the corresponding aldehyde[15]. The proposed criterion for the consideration of the kinetic importance of ternary complexes is simpler than the criterion used in conventional diagnostics (*i.e.* a comparison of the rate constants ratios[15]). The data obtained with the proposed system using NDMA are more reliable than the data measured in the classical way, where NADH concentration changes are measured. The reaction rate in the system with NDMA is substantially higher and the absorption coefficient of NDMA is about 5·7 times as high as that of NADH.

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