ANALYSIS OF ONE ENZYME RECYCLIZATION SYSTEM WITH LOW ENZYME CONCENTRATION

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One enzyme recyclization reactions in the presence of two substrates and a small amount of a coenzyme are characterized by a long prestationary phase, in the course of which the distribution of coenzyme forms varies. The kinetics of the coenzyme prestationary phase was analyzed theoretically and some equations characterizing mainly the start and the end of this phase were derived. To verify experimentally the derived equations two recyclization systems with horse liver alcohol dehydrogenase, NAD or NADH, and corresponding substrate pairs (*p*-nitrosodimethylaniline and 1-butanol, or furyl alcohol and acetaldehyde) were investigated. Consequences for the analytical practice and utilization of these reactions for the determination of small coenzyme and enzyme concentrations are discussed.

For the determination of some coenzyme sensitive recyclization reactions have been used based on the catalytic action of one or two specific enzymes on the corresponding substrates in the presence of catalytic quantities of coenzymes[1-4]. A typical example is the measurement of NAD concentration using NAD-dependent dehydrogenases. The recyclization methods proposed can be used in principle also for the determination of very low activities of respective enzymes[5-7].

When "one enzyme" recyclization reactions are performed in the usual way, the enzyme and the coenzyme are present in relatively very low concentrations, whereas substrates are used in concentrations as high as possible. The measured reaction rate is settled in a definite time at a constant value proportional to the concentration of the enzyme and the coenzyme.

The aim of this paper is to treat the establishment of the steady-state and to discuss some kinetic properties of the one enzyme recyclization systems. The validity of the general equations derived is verified experimentally using two recyclization systems with horse liver alcohol dehydrogenase (ADH).

EXPERIMENTAL

Material and Methods

ADH was isolated as described earlier[8], molar concentration of enzyme active centers was determined from its activity[8]. Coenzymes NAD and NADH (grade I) were from Boehringer (Mannheim), *p*-nitrosodimethylaniline (NDMA) was synthesized[8], furyl alcohol (International Enzymes) was purified as described earlier[7], further reagents were of analytical grade.

Spectrophotometric measurements were made using Cary 118 apparatus in a 0·1M sodium-phosphate buffer, pH 8·5, at 25°C. Reaction rates were determined from the decrease of NDMA absorbance[6] (ε_{440} 35·4 $mM^{-1}\ cm^{-1}$, velocity v_1), increase of fural absorbance[7] (ε_{280} 14·5 $mM^{-1}\ .\ cm^{-1}$, velocity v_2) and from the changes of NADH absorbance (ε_{340} 6·2 $mM^{-1}\ cm^{-1}$, velocity v_3). Reactions were started by adding small amounts of the enzyme.

RESULTS AND DISCUSSION

The simplest variant of one enzyme recyclization reaction can be described by a scheme (*1*) based on the validity of Theorell–Chance mechanism for substrates S_1^0 and S_2^r. Theorell–Chance approximation assumes kinetic negligibility of ternary complexes $EC^rS_1^0$ and $EC^0S_2^r$

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

E represents the enzyme, C^0 and C^r are two forms of the coenzyme (NAD and NADH, for instance), S_1^0 and S_2^r are corresponding substrates (for instance aldehyde and alcohol in the case of ADH) and k are rate constants. The reaction is followed in the initial phase, in which respective products S_2^0 and S_1^r do not interfere. As a recyclization reaction proper is considered the rapid interconversion $EC^0 \rightleftharpoons EC^r$.

Let us assume that the initial concentration of the coenzyme forms is very low and that the initial concentration of substrates S_1^0 and S_2^r is much higher than the total coenzyme concentration ($C_{tot} = C^0 + C^r$). Total enzyme concentration E_{tot} is still much lower than C_{tot}. It holds S_1^0, $S_2^r \gg C_{tot} \gg E_{tot}$ (*2*). During the observed reaction course mainly the distribution of C^0 and C^r is changed, the relative decrease of S_1^0 and S_2^r being negligible. The same symbols are thus used for initial concentrations as for the concentrations at any arbitrary time t. Generally one can measure the changes of concentrations S_1^0, S_2^r and C^r with time, *i.e.* the velocities v_1, v_2 and v_3 that can be expressed by equations (*3*). (If the changes of concentrations S_1^r, S_2^0 and C^0 are followed the corresponding equations are identical except for the sign.)

$$\begin{aligned}
v_1 &= -dS_1^0/dt = E_{tot}/D \,.\, \left[(k_1C^0 + k_2C^r)\, k_{-3}k_4S_1^0S_2^r + k_{-1}k_2k_{-3}C^rS_1^0\right] \\
v_2 &= -dS_2^r/dt = E_{tot}/D \,.\, \left[(k_1C^0 + k_2C^r)\, k_{-3}k_4S_1^0S_2^r + k_1k_{-2}k_4C^0S_2^r\right] \\
v_3 &= \quad dC^r/dt = E_{tot}/D \,.\, (k_1k_{-2}k_4C^0S_2^r - k_{-1}k_2k_{-3}C^rS_1^0) = v_2 - v_1 \\
D &\approx k_{-1}k_{-2} + k_{-1}k_{-3}S_1^0 + k_{-2}k_4S_2^r \qquad (3)
\end{aligned}$$

In the denominator D only the terms not containing C^0 or C^r are taken into account, for the concentration of both coenzyme forms is assumed to be very low.

On starting the reaction by adding the enzyme a short enzyme prestationary phase takes place first, but soon an enzyme steady state becomes established, sooner than any significant change in the distribution of coenzyme forms occurs (compare Eq. (2)). The velocities corresponding to this phase are the initial velocities $(v_1)_0$, $(v_2)_0$ and $(v_3)_0$. This enzyme steady state is kept in the further course of the reaction and represents an initiation of the prestationary phase with respect to the coenzyme, in which changes of the distributions of C^0 and C^r occur. In the last reaction phase considered the coenzyme steady state is established characterized by a relatively constant steady state velocity v_s

$$(v_1)_s = (v_2)_s = v_s\,, \quad (v_3)_s = 0\,. \tag{4}$$

If the initial velocity $(v_1)_0$ or $(v_2)_0$ in the presence of both coenzyme forms should be equal to the velocity after establishing the coenzyme steady state v_s and the initial velocity $(v_3)_0$ should be equal to zero, then following equation for the initial concentrations of the coenzyme forms $(C^r)_0$ and $(C^0)_0$ must hold $(C^r)_0/(C^0)_0 = Q^r/Q^0$ (5), where $Q^r = k_1 k_{-2} k_4 S_2^r$ and $Q^0 = k_{-1} k_2 k_{-3} S_1^0$. Thus at definite initial concentrations S_1^0 and S_2^r and $(C^0)_0$ only one initial concentration $(C^r)_0$ exists, for which $(v_1)_0 = (v_2)_0 = v_s$, $(v_3)_0 = 0$.

The coenzyme steady state is characterized by following distribution of the total coenzyme concentration C_{tot} between the forms C^0 and C^r

$$(C^0)_s = C_{tot}\,.\,Q^0/(Q^0 + Q^r)$$

$$(C^r)_s = C_{tot}\,.\,Q^r/(Q^0 + Q^r)\,. \tag{6}$$

Comparing the initial velocities $(v_1)_0$ and $(v_2)_0$ under conditions when only one of the coenzyme forms is present at the onset of the reaction (thus $(v_1)_0\,(C^r = 0)$, $(v_1)_0\,(C^0 = 0)$, $(v_2)_0\,(C^r = 0)$ and $(v_2)_0\,(C^0 = 0)$) with the velocity achieved after the coenzyme steady state was established (v_s), we obtain following expressions

$$(v_1)_0\,(C^0 = 0) > v_s > (v_1)_0\,(C^r = 0)\,, \quad \text{if} \quad k_{-1}k_2 > (k_1 - k_2)\,k_4 S_2^r \tag{7}$$

$$(v_2)_0\,(C^r = 0) > v_s > (v_2)^0\,(C^0 = 0)\,, \quad \text{if} \quad k_1 k_{-2} > (k_2 - k_1)\,k_{-3} S_1^0\,. \tag{7'}$$

If $k_1 > k_2$ (*i.e.* the rate constant for C^0 association with the enzyme is greater than the constant for C^r association), inequality (7') is valid and a definite critical concentration S_2^r exists, which determines the relation between the velocities $(v_1)_0$ and v_s. If the actual initial concentration S_2^r is smaller than this critical value, inequality (7) is valid.If the initial concentration S_2^r reaches the critical value, all velocities in equation (7) are equal. If S_2^r is greater than the critical value of concentration

it holds $(v_1)_0 (C^0 = 0) > v_s > (v_1)_0 (C^r = 0)$. On the other hand if $k_2 > k_1$, inequality (7) is always satisfied, whereas equation (7′) is valid only in the case of S_2^r being smaller than the corresponding critical concentration. If the critical concentration is reached, the velocities $(v_2)_0$ and v_s are equal, if it is exceeded inverted signs are valid in inequality (7′). If $k_1 = k_2$ both inequalities (7) and (7′) are valid.

If we are interested not only in the extreme states of the recyclization one enzyme system with a very low coenzyme concentration (*i.e.* in the state when the steady-state distribution of the enzyme forms only is established, and in the coenzyme steady state), but if we want to characterize also the transition between both phases, we have to formulate the time course of the concentration of one of the reacting compounds. The time dependence of the substrate concentration S_1^0 is described by following equations (in Eq. (8) only form C^0 is present at the start, in Eq. (8′) only form C^r)

$$\Delta S_1^0 = -\alpha C_{tot} E_{tot} \cdot t + Q^r \beta C_{tot} [1 - \exp(-\gamma E_{tot} t)] \quad \text{for} \quad (C^0)_0 = C_{tot} \qquad (8)$$

$$\Delta S_1^0 = -\alpha C_{tot} E_{tot} \cdot t - Q^0 \tau \beta C_{tot} [1 - \exp(-\gamma E_{tot} t)] \quad \text{for} \quad (C^r)_0 = C_{tot} \qquad (8')$$

where ΔS_1^0 is the difference between the initial substrate concentration S_1^0 and its value at time t

$$\alpha = k_1 k_2 k_{-3} k_4 S_1^0 S_2^r / (Q^0 + Q^r), \quad \beta = (k_{-1} k_2 k_{-3} S_1^0 + k_2 k_{-3} k_4 S_1^0 S_2^r - k_1 k_{-3} k_4 S_1^0 S_2^r) / (Q^0 + Q^r)^2, \quad \gamma = (Q^0 + Q^r)/D .$$

The corresponding initial and steady-state rates $(v_1)_0 (C^r = 0)$, $(v_1)_0 (C^0 = 0)$ and v_s can be obtained by the differentiation of equations (8) and (8′) for $t = 0$ and $t \to \infty$, respectively. They are identical to the velocities calculated from equations (3) obtained on substituting for C^0 and C^r the initial values $(C^0)_0 = C_{tot}$, $(C^r)_0 = C_{tot}$ or the steady-state values $(C^0)_s$ and $(C^r)_s$ from equations (6)

$$(v_1)_0 (C^r = 0) = (\alpha - Q^r \beta \gamma) C_{tot} E_{tot}, \qquad (9)$$

$$(v_1)_0 (C^0 = 0) = (\alpha + Q^0 \beta \gamma) C_{tot} E_{tot},$$

$$v_s = \alpha C_{tot} E_{tot} .$$

It follows from the equations (8) and (8′) that the transition to the steady-state for the coenzyme is similar to the establishment of the steady-state distribution of the enzyme forms in the classical prestationary kinetics – the time change of the S_1^0 concentration is a function containing a linear and an exponential term. The general graphical form of these functions is illustrated in Fig. 1. Fig. 1 shows also the graphical determination of the two main experimental characteristics of the prestationary

phase, namely of the intercepts q intersected on the ΔS_1^0 axis by the extrapolated linear parts of the functions obtained ($q^0 = -Q^0\beta C_{tot}$ and $q^r = Q^r\beta C_{tot}$ according to (8) and (8')) and of the transient time τ, *i.e.* of the time in which the experimental curves intersect the lines through points $-q^0/2$ and $q^r/2$ at the ΔS_1^0 axis parallel to the linear parts of the functions (Fig. 1). According to Eq. (8) and (8') these

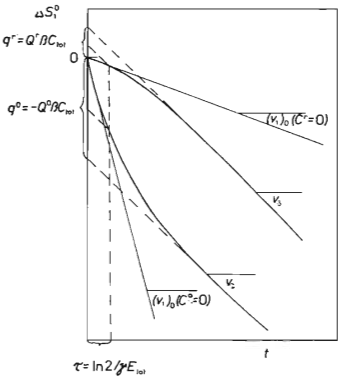

FIG. 1

An example of the dependence of substrate concentration decrease (ΔS_1^0) at a low coenzyme concentration C^0 (upper curve) and C^r (lower curve) on time (t). The evaluation of six parameters characterizing the prestationary and the steady-state phase for the coenzyme is indicated: initial velocities $(v_1)_0$ ($C^0 = 0$) and $(v_1)_0$ ($C^r = 0$), steady-state velocity v_s, transient time (τ) and the intercepts at the ΔS_1^0 axis (q^0 and q^r). The curves were generated according to (8) and (8') for $Q^0 = 3$ and $Q^r = 1$

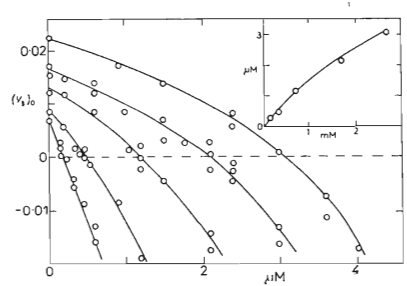

FIG. 2

Initial NADH conversion rate ($(v_3)_0$) as a function of the initial NADH concentration ($(C^r)_0$) for low 1-butanol concentrations (S_2^r). Concentration of the reaction mixture components [NDMA] 60 μM (S_1^0), [NAD] 15 μM ($(C^0)_0$), [ADH] 4 nM (E_{tot}), 1-butanol concentrations 0·16, 0·33, 0·67, 1·7 and 2·7 mM. In the upper part initial NADH concentrations (μM), for which holds $(v_3)_0 = 0$ under given conditions, are plotted against butanol concentration (S_2^r)

characteristics should differ in their dependence on the concentration variables C_{tot} and E_{tot}: intercepts q^0 and q^r on ΔS_1^0 axis are directly proportional to the initial concentrations $(C^r)_0$ and $(C^0)_0$ (equal to C_{tot}), respectively, and are independent of E_{tot}, whereas the reciprocal transient time is a linear function of E_{tot} $(1/\tau = E_{tot} : : \ln 2)$. In addition, the absolute value of the ratio of the intercepts intersected on the ΔS_1^0 axis is identical to expression (5) derived for the ratio of the initial concentrations of the coenzyme forms if the condition $(v_1)_0 = (v_2)_0 = v_s$ is satisfied.

The equations and the predictions derived can be used as criteria for testing the applicability of the suggested model of the one enzyme recyclization reaction in the presence of a low coenzyme concentration. To verify the presented equations the behavior of two systems containing horse liver alcohol dehydrogenase and low concentrations of NAD (C^0) and NADH (C^r), respectively, was investigated. In the first system the substrate S_1^0 was represented by *p*-nitrosodimethylaniline (NDMA) substituting an aldehyde, 1-butanol was the substrate S_2^r. In the second system furyl alcohol (S_2^r) and acetaldehyde (S_1^0) were used and the amount of the fural formed was measured (v_2).

The concentration conditions present if the initial velocity $(v_1)_0$ in the presence of both coenzyme forms is equal to the velocity reached in the coenzyme steady state are illustrated in Fig. 2. It is demonstrated that in the case of the first system equation (5) holds exactly at least for lower butanol concentrations (S_2^r). Relatively high NAD concentrations had to be used (only four times lower than the NDMA concentration) to obtain conveniently measurable initial velocities. Under given conditions it holds $Q^r \ll Q^0$ – compare Eq. (5).

Concerning the comparison of initial velocities with the steady-state velocities (v_s) under the conditions of one coenzyme form only present at the onset of the reaction, both systems mentioned were tested. It was found that in the system containing

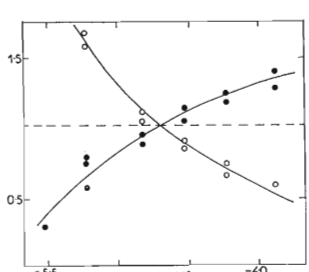

FIG. 3

Dependence of the ratio of initial and steady-state velocity $(v_2)_0/v_s$ on the logarithm of acetaldehyde concentration (S_1^0). Concentrations of the reaction mixture components [furyl alcohol] 3·5 mM (S_2^r), [ADH] 13 nM (E_{tot}), (○)[NAD]0·3 μM $(C^0)_0)$, (●)[NADH] 0·3 μM $((C^r)_0)$

NDMA inequality (7) is always valid, *i.e.* $(v_1)_0\,(C^0 = 0) > v_s > (v_1)_0\,(C^r = 0)$, because the rate constant for NADH association with ADH is higher[9] than that for NAD association ($k_2 > k_1$). The behavior of the system with furyl alcohol is shown in Fig. 3. The system behaves as anticipated (comp. Eq. (7')). For low acetaldehyde concentrations (S_1^0) the initial velocity in the presence of NAD is higher than the steady-state velocity, which is, in turn, higher than the initial velocity in the presence of NADH. Hence it holds $(v_2)_0\,(C^r = 0) > v_s > (v_2)_0\,(C^0 = 0)$. For an acetaldehyde concentration of about 25 μM all three velocities are equal, for acetaldehyde concentrations higher than this critical concentration it holds $(v_2)_0\,(C^0 = 0) > v_s > (v_2)_0\,(C^r = 0)$.

The establishment of the coenzyme steady state was studied for the system with NDMA and dependences similar to those shown in Fig. 1 were obtained. In the presence of low initial NADH concentrations under given conditions, the effects observed were more pronounced than in the presence of NAD. This is related to the condition $Q^r \ll Q^0$ (comp. Eq. (5)) and is in accordance with the behavior of this system in the presence of both coenzyme forms at the start of the reaction as already described.

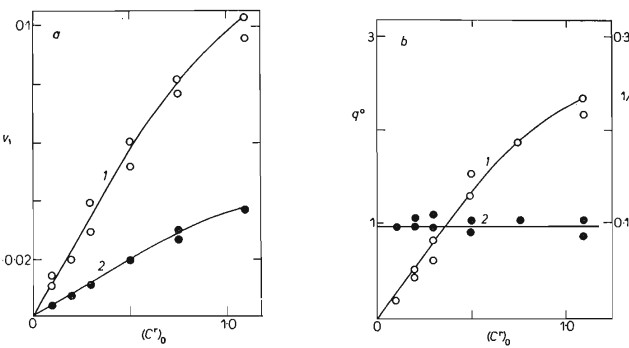

FIG. 4

Parameters of the prestationary and steady-state phase for the coenzyme as a function of the initial NADH concentration ($(C^r)_0$). Concentrations of reaction mixture components [NDMA] 40 μM (S_1^0), [1-butanol] 2·5 mM (S_2^0), [ADH] 8 nM (E_{tot}). The parameters were evaluated as in Fig. 1. *a* (1) initial velocity $(v_1)_0\,(C^0 = 0)$, (2) steady state velocity v_s, *b* (1) parameter q^0, (2) reciprocal transient time $(1/\tau)$

The transition between the enzyme and coenzyme steady state phase was measured as a function of NADH concentration ($(C^r)_0 = C_{tot}$) and enzyme concentration E_{tot}. The results are summarized in Figs 4 and 5. The dependence of the initial velocity $(v_1)_0$ $(C^0 = 0)$ and of the steady-state velocity v_s on NADH concentration is linear under given conditions approximately up to the concentration of 0·5 μM (Fig. 4*a*). The respective dependences on ADH concentration are linear in the whole interval 0–12 nM (Fig. 5*a*). The system behaves as anticipated as far as the dependences of the intercept on the ΔS_I^0 axis (q^0) and the transient time (τ) on the enzyme and coenzyme concentrations are concerned (compare Eq. (*8*) and (*8'*) and (Fig. 1). The negligible increase of q^0 as a function of E_{tot} may be due to the contamination of the enzyme preparation by traces of NADH. On the other hand the value of the reciprocal transient time $(1/\tau)$ does not depend on the enzyme concentration (Fig. 4*b*), but is proportional to the concentration of the alcohol dehydrogenase (Fig. 5*b*) – in agreement with the equation $1/\tau = \gamma E_{tot}/\ln 2$.

For practical analytical purposes, *i.e.* for the determination of coenzyme or enzyme concentrations from the reaction rate, the steady-state velocity with respect to the coenzyme (v_s) is usually the starting point. Although this velocity can be substantially lower than the initial velocity (as it is in Figs 4*a* and 5*a*), it can be evaluated much more exactly from the linear part of the registered dependence (comp. Fig. 1). Using the enzyme concentration as high as possible is advantageous for the evaluation of the coenzyme concentration. In this case a higher v_s value is obtained and the prestationary phase is significantly shorter (under given conditions in the system

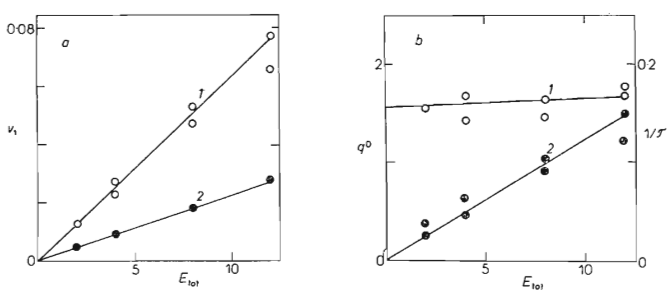

FIG. 5

Parameters of the prestationary and steady-state phase as a function of ADH concentration (E_{tot}). Symbols and conditions of measurements see Fig. 4, [NADH] 0·5 μM ($(C^r)_0$)

with NDMA the concentration of ADH higher than 50 nM makes it safe that the transient time of this phase will be shorter than 2 s).

The determination of the coenzyme concentration v_s does not allow to distinguish the coenzyme forms C^0 and C^r and only the sum of their concentrations can be obtained (the velocity v_s is the same for both forms (comp. Eq. (9)). Short time measurements in the presence of a low coenzyme concentration (when coenzyme prestationary phase can still be detected) could help under suitable conditions to distinguish the respective coenzyme form or at least to indicate the coenzyme form present in higher quantity.

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