STUDIES ON THE INTERACTION OF GLUTAMATE DEHYDROGENASE WITH APORHEINE

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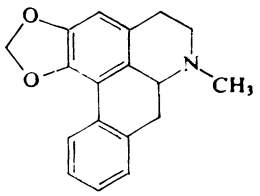
The effect was examined of two inhibitors, aporheine and thyroxine, on the kinetics of action of glutamate dehydrogenase in the presence of the coenzyme, NADH, in an inhibiting excess. From the results obtained it appears probable that these two competing ligands do not bind to the regulatory binding site of the enzyme. If the good substrate, glutamate, is replaced by a poor one, such as alanine, aporheine can behave under certain conditions as an activator; this phenomenon is most likely due to the fact, like in other cases, that the association of alanine with the enzyme-coenzyme complex is the slowest step of the reaction. The inhibitory action of aporheine in alkaline media is more complicated than its action at neutral and acidic pH. The sigmoid dependence of inhibition on aporheine concentration is best interpreted as a result of the induction of the second binding site of the enzyme subunit for aporheine after the binding of the first molecule of this ligand. The results obtained indicate the presence of a positive charge localized in the neighborhood of the binding site for the first aporheine molecule and also the important role played by some group of the enzyme with a pK-value about 8 during the induction of the second binding site with a relative high affinity for this ligand.

It has been observed[1] that certain isoquinoline alkaloids are capable of binding to glutamate dehydrogenase (E.C. 1.4.1.2, GDH) and thus inhibit the activity of the enzyme. The strongest inhibitor of these alkaloids so far tested is aporheine (*I*).



I

An efficient binding of this inhibitor to the enzyme occurs only after the coenzyme has been bound to the active site of the enzyme subunit. Aporheine most likely binds to the binding site of GDH for thyroxine which acts as an effector. The inhibitory power of aporheine and other alkaloids of the isoquinoline type is considerably higher at pH-values higher than the physiological pH 7 (ref.[1]).

The aim of this study has been to analyze in more detail the effect of aporheine on the inhibition of GDH by an excess of NADH with respect to a more exact location of the enzyme binding site for isoquinoline alkaloids; an effort has been also made to analyze the effect of this inhibition in the presence of another substrate (alanine) and especially to examine the inhibitory action of aporheine at alkaline pH in detail.

EXPERIMENTAL

Aporheine was donated by Professor J. Slavík, Purkyně University, Brno, NAD and NADH were from Boehringer (Mannheim, FRG), thyroxine from Reanal (Hungary), α-oxoglutaric acid from J. T. Baker Chem. (Deventer, Holland). The remaining chemicals were purchased from Lachema (Czechoslovakia).

Bovine liver glutamate dehydrogenase was from Boehringer (Mannheim, FRG); its activity and concentration were measured as described elsewhere[1]. The enzyme concentration is given in molar concentrations of subunits of the functional hexamer. E stands for enzyme subunit (or concentration of enzyme subunits).

The kinetic measurements were made in Cary 118 spectrophotometer at 25°C as described before[1]. The initial reaction rates were determined from increments of absorbance measured at 340 nm and are expressed in relative units.

The inhibitory influence of aporheine (I) at various pH was evaluated in a computer. The method of nonlinear regression analysis based on the Marquardt algorithm[2] was used. The relative standard deviations of the parameter (inhibition constants) were calculated from the inverted set of variants-covariants[2]; the quality of the calculation was judged by the relative residual sum of squares[2]. The appropriateness of the model (either $i = I/(K_1 + I)$ or $i = (I/K_1 + I^2/K_1K_2)/(1 + I/K_1 + I^2/K_1K_2)$ was considered with respect to the above characteristics, by visual comparison of the measured and calculated values, and by the sign test[3].

The fluorescence polarization was measured and evaluated as described in ref.[1].

RESULTS AND DISCUSSION

Effect of Aporheine and Thyroxine in the Presence of ADP *and of an Excess of* NADH

The coenzyme, NADH, when present in high concentrations acts as an inhibitor of GDH (ref.[4]). Under these conditions it binds both to the active center of the enzyme and also to the so-called regulatory (allosteric) site of the GDH subunit[4]. This regulatory site has an affinity especially for the activator, ADP (ref.[4]). The effect of aporheine on the enzyme activity of GDH during its binding to the enzyme competes with the effect of ADP (aporheine inhibits, ADP activates; the activating effect of ADP prevails in the presence of high concentrations of both these effectors[1]. The behavior of ADP towards thyroxine is similar; the elimination of the inhibitory effect of thyroxine by an excess of ADP interprets Wolff[5] as evidence of the binding of thyroxine to the regulatory site of GDH, whose role is to bind ADP and the second

molecule of NADH. This interpretation is not, however, entirely adequate: it cannot be concluded that ADP and thyroxine compete from the mere fact that the inhibitory effect of thyroxine does not manifest itself at saturating concentration of ADP. The character of the dependence of the initial reaction rate on ADP concentration in the presence of both thyroxine and aporheine[1] does not eliminate the possibility of formation of enzyme complexes with ADP and thyroxine or aporheine bound simultaneously. (The activating effect of ADP will suppress the inhibitory effect of thyroxine or aporheine.) Fig. 5 in ref.[1], which describes the effect of aporheine on the activity of GDH in the presence of ADH, even suggests the possibility of mutual stabilization of the bond of ADP and aporheine to GDH. A piece of direct evidence of this stabilizing effect represents the increase of fluorescence polarization of aporheine (λ_{exc} 320 nm, λ_{em} 380 nm) in the presence of GDH after the addition of ADP. ADP was added (final concentration 0·2 mmol/l) to a mixture of 30 μmol/l GDH and 10 μmol/l aporheine whose polarization was about 0·05. The fluorescence polarization of the resulting mixture was 0·10–0·11. The stabilizing effect of ADP on aporheine binding is slightly lower than the effect of the coenzyme (NAD, whose addition increased the polarization to a value close to 0·15 under identical conditions, *cf.* ref.[1]), yet it clearly shows that the formation of a ternary complex GDH subunit-aporheine-ADP is possible. It was impossible to carry out an analogous experiment with thyroxine since this compound does not show the proper fluorescence characteristics.

Likewise, a more detailed analysis of the effect of aporheine and thyroxine on the inhibition of GDH activity by an excess of NADH is in disagreement with the postulate[5,6] that the binding sites for these ligands are identical with the regulatory binding site of the GDH subunit for ADP and the second NADH molecule. The kinetic model of the interaction of NADH (occupying both the active site and the regulatory binding site) with another ligand (*i.e.* thyroxine or aporheine) can be analyzed, to a first approximation, by the method of partial equilibrium on assumption that a rapid equilibrium between the individual enzyme forms is established and that the NAD formed dissociating from the active site is the step limiting the sequence of the reaction[6] (*cf.* Supplement).

In experiments with inhibitors of aporheine type (*i.e.* noncompetitive with respect to NADH and thus requiring the presence of NADH in the active center for an effective binding) the position of the maximum of the profile representing the dependence of reaction rate on NADH concentration should not vary if there were competition of the inhibitor with the second NADH molecule. A shift of the maximum toward lower NADH concentrations should occur only if complexes with two NADH molecules and one inhibitor molecule were formed. On the other hand, an even stricter requirement can be derived, on the above assumption, for inhibitors of the thyroxine type (*i.e.* noncompetitive with respect to the coenzyme, inhibitors whose affinity is not affected any substantially by the presence of the coenzyme in the

active center). In this case it should hold that the inhibitor and the second NADH molecule bind without affecting each other (noncompetitive binding of the inhibitor and of the second NADH molecule) only if the position of the maximum is unchanged by the presence of the inhibitor. A shift of the maximum toward lower values signalizes in this case the stabilization of the binding of the inhibitor and of the second NADH molecule (*cf.* Supplement).

A confrontation of these theoretical considerations with the measured data (Fig. 1) also supports the assumption that the binding site for thyroxine and aporheine is not identical with the binding site of the enzyme for the second NADH molecule acting as an inhibitor (and for the activator, ADP). It is likely that the complexes of GDH with the occupied (by ADP or NADH molecules) regulatory site and with the occupied binding site for thyroxine and isoquinoline alkaloids can form and that the influence of the allosteric effector in the regulatory site will become prevalent (*e.g.*, in case of ADP the labilizing effect on the binding of the coenzyme will prevail over the stabilizing effect of aporheine; the resulting stabilization of the coenzyme in the active center leads to an increase in reaction rate, *cf.* ref.[7] and below).

Effect of Aporheine on the Kinetics of GDH *in the Presence of Alanine*

Alanine is a considerably worse substrate of GDH than its "natural" substrate, *i.e.* glutamate[7]. The behavior of glutamate toward aporheine is practically noncompetitive[1]. If, however, glutamate is replaced by alanine the effect of aporheine on the reaction rate changes: inhibition can be observed at high concentrations and activation at low concentrations (Fig. 2). This transition from inhibition to activation as a result of replacement of a good substrate (glutamate) by a poor one (alanine) can be explained by assuming that the slowest rate limiting steps of the enzymatic reaction catalyzed by GDH vary with the individual substrates. GDH acts as an enzyme with ordered mechanism of action: the coenzyme is bound as the first one to the enzyme subunit and next follows the substrate. The sequence is the opposite during dissociation. It is known[7] that the slowest step in the reaction sequence in the presence of glutamate is the dissociation of the reduced coenzyme formed by the reduction of oxidized coenzyme NAD on account of glutamate. On the other hand, if a poor substrate, alanine, is present, the slowest step in the reaction sequence is the binding of the substrate to the E–NAD complex[7]. If both substrates are used in the presence of aporheine unproductive complexes of GDH with aporheine are formed which besides the inhibitor also involve the coenzymes and the substrates; this phenomenon is due to the fact that aporheine acts as a total GDH inhibitor. (The initial reaction rate in the presence of high aporheine concentrations approximates zero[1].) The formation of these inactive complexes of GDH with aporheine is, in addition to the stabilization of the coenzyme binding, the basic reason why this compound acts as an inhibitor if both substrates are used. If, however, alanine is used as a substrate

(at low concentrations) another effect probably plays a role. Since the binding of alanine to the E–NAD complex is the slowest phase of the reaction sequence, this step may be speeded up by the presence of aporheine. The binding of aporheine namely facilitates the binding of the coenzyme and most likely also enhances the association of the coenzyme and the substrate with the active center of GDH (ref.[1]). It is therefore likely that the sequence E→E-NAD→E-NAD-alanine is slower

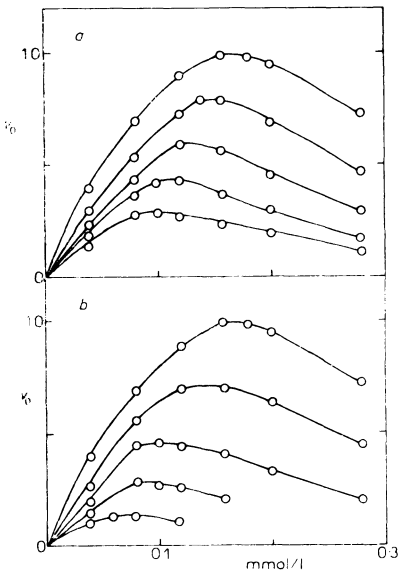


FIG. 1

Effect of aporheine (*a*) and thyroxine (*b*) on inhibition of GDH by excess of NADH. The reactions were carried out in 0·1 mol/l Na-phosphate buffer, pH 7·5, containing 2 mmol/l α-oxoglutarate, 50 mmol/l NH_4Cl, and 0·1 mmol/l EDTA. The reactions were triggered by a small addition of the concentrated enzyme solution to a final concentration of 15 nmol/l. mmol/l concentration of NADH in reaction mixture, v_0 relative initial reaction rate (the rate in the maximum is regarded as equaling one), *a* aporheine concentration corresponding to the individual curves (from the top): 0, 0·1, 0·15, 0·2, 0·3 mmol/l), *b* thyroxine concentration corresponding to the individual curves (from the top): 0, 5, 10, 20, 30 μmol/l

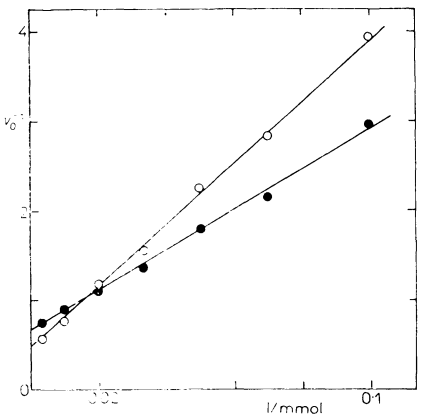


FIG. 2

Inhibitory and activating effect of 0·1 mmol/l aporheine (●) in presence of alanine as substrate. (○) Comparative measurement in the absence of inhibitor. The reactions were carried out in 0·1 mol/l NaOH–glycine buffer, pH 9 containing 4 mmol/l NAD and 0·1 mmol/l EDTA and triggered by the addition of GDH to a concentration of 60 nmol/l. v_0^{-1} reciprocal relative initial reaction rate (its value at an alanine concentration at which the reaction rates in the presence and absence are equal, is considered being one), 1/mmol reciprocal alanine concentration

that the sequence E→E-NAD→E-NAD-aporheine→E-NAD-aporheine-alanine →E-NAD-alanine if the concentration of alanine in the reaction medium is low. A similar effect after the replacement of glutamate by alanine (and, likewise, an analogous explanation) have been reported[7] in the case of GTP which represents another specific GDH inhibitor. This inhibitor, however, binds to a site of the GDH subunit different from that, where the isoquinoline alkaloids are bound. When analyzing the reverse reaction, *i.e.* NADH oxidation, a similar effect has been observed also in the case of thyroxine whose binding site on GDH is most likely identical with the binding site for the isoquinoline alkaloids (see above). If the "natural" substrate, α-oxoglutarate, is replaced by pyruvic acid (*i.e.* a product of oxidative alanine deamination during the action of GDH), activation can also be observed in the presence of low thyroxine concentrations.

Inhibitory Effect of Aporheine in Alkaline Media

The inhibition of GDH by isoquinoline alkaloids is considerably stronger in alkaline media than in acidic and neutral media. As can be seen from Fig. 3 the inhibitory action of aporheine under these conditions is a little more complicated. Unlike in neutral and weakly acidic media, where the dependence of inhibition on concentration can be represented by the usual hyperbolic curve, a sigmoidal profile can be observed at higher pH-values (Fig. 3). This phenomenon can be the result in principal of several causes which will be discussed.

a) Sigmoidal profiles, analogous to the curve observed at pH 9 (Fig. 3) can be obtained under certain conditions in the presence of some partial, noncompetitive inhibitors; in such cases the complexes of the enzyme containing the substrate and the inhibitor show enzymatic activity, *i.e.* can dissociate to the product[1]. This explanation, however, cannot be applied in our case since the enzymatic activity approaches zero at high aporheine concentrations (Fig. 3 and ref.[1]); hence, aporheine acts as a total inhibitor.

b) Similar sigmoidal curves can be observed sometimes also with enzymes which can form aggregates of different affinity for the given ligand (the so-called polymerization model, *cf.* ref.[8]). The association of functional GDH hexamers to aggregates at higher concentrations of the protein has been described[4]. If this mechanism were effective then the inhibitory effect should vary with the various enzyme concentrations (the equilibria between the individual aggregation degrees being affected[8]). Since profiles very similar to those shown in Fig. 3 can also be observed over a very wide range of GDH concentrations (5–200 nmol/l), even this explanation of the effect observed is unacceptable.

c) The most probable explanation of the described sigmoidal profile is the classical concept of positive cooperativity between at least two binding sites of the enzyme

for the ligand (*i.e.* of GDH for aporheine) with a different affinity for this ligand. Since the induction of cooperativity between the enzyme subunits as a result of a change in pH seems improbable we shall make an effort to explain these phenomena by postulating the existence of two binding sites for aporheine on each GDH subunit.

The behavior observed can be described in the simplest manner by a model which represents a general analogy to the model of Koshland, Némethy, and Filmer[9]

$$E \overset{K_1}{\rightleftharpoons} EI \overset{K_2}{\rightleftharpoons} EI_2$$

with two inhibition (dissociation) constants K_1 and K_2, where $K_1 > K_2$. It is necessary that the first binding site be occupied for aporheine (*i.e.* inhibitor *I*) to bind

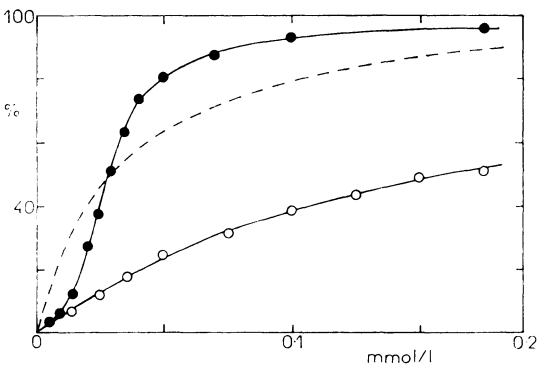


FIG. 3

Inhibitory effect of aporheine (mmol/l) at pH 7 and 9. The buffers used were 0·1 mol/l Na-phosphate, pH 7 (○) and 0·1 mol/l NaOH-glycine, pH 9 (●). The composition of the reaction mixtures and the conditions of the measurement were analogous to those shown in Fig. 1, % relative inhibition calculated from the formula $i = (1 - v_i/v_0)$. . 100, (-----) hypothetic dependence of inhibition on aporheine concentration as if the plot were hyperbolic even at pH 9 (assuming a K_1-value of 30 μmol/l)

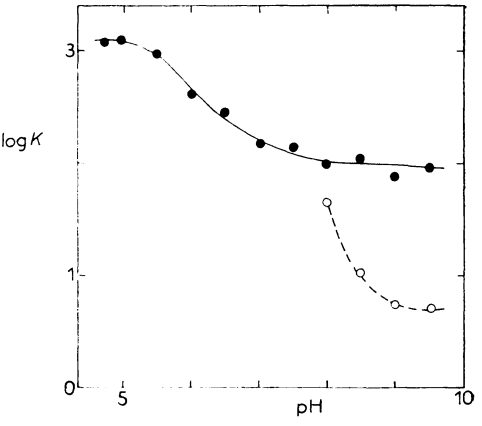


FIG. 4

Dependence of logarithms of inhibitory constants K_1 (●) and K_2 (○) on pH. The measurements were carried out as shown in Fig. 3. The buffers used were 0·1 mol/l acetate, pH 4·8—6·0, 0·1 mol/l Na-phosphate, pH 6·0—8·0, and 0·1 mol/l NaOH-glycine, pH 8·0—9·5. The results of parallel experiments at pH 6 and 8, where various buffers were used, were practically identical. The inhibition constants were calculated in a computer; the hyperbolic relation with one inhibition constant K_1 was more suitable for acidic and neutral media, the cooperative relation with constants K_1 and K_2 was more suitable for alkaline media (*cf.* Methods).

to the second binding site, even though the binding of the inhibitor to the latter site is stronger. It can be assumed that K_2 is very high in acidic and neutral media and the association of aporheine with the second binding site is practically impossible. It remains to be shown whether complexes EI and EI_2 (representing in this very simple system of symbols all forms of the GDH subunit with one or two, resp. bound molecules of the inhibitor, *i.e.* also the forms containing bound coenzymes and substrates) are enzymatically active or not. Since aporheine is a total inhibitor at neutral and acidic pH, where complexes of the EI type only are formed, it is likely that the complexes of this type are inactive. Since practically no enzyme activity is observed after GDH has been saturated at pH 9 with aporheine (Fig. 3) it may be assumed that complexes of the EI_2 type are not enzymatically active either.

On assumption that no form of the enzyme containing bound aporheine is active, *i.e.* that form E only is enzymatically active, the inhibition (i) in the presence of aporheine (I), defined by the formula $i = 1 - v_i/v_0$, where v_i is the initial reaction rate in the presence of the inhibitor and v_0 in the absence of the inhibitor, can be expressed by the formula $i = (I/K_1 + I^2/K_1K_2)/(1 + I/K_1 + I^2/K_1K_2)$. The dependence of the inhibition on aporheine concentration (function *i versus I*) was measured for at least ten I, i pairs at various pH-values. The data obtained, similar to those shown in Fig. 3, were computer-treated on assumption of the validity of both the hyperbolic and the sigmoidal model (*cf.* Methods). According to the criteria given under Methods the single hyperbolic model better describes the inhibitory effect of aporheine in acidic and neutral media whereas the sigmoidal model considerably better reflects the inhibition in alkaline media. Plots of relatively good quality were obtained with the appropriate models at individual pH-values (the relative residual sums of the squares were low) and also the agreement of the plots with these simple equations was good (the sign test was negative). Similarly, the parameters obtained (*i.e.* the inhibition constants K_1 or pairs of constants K_1K_2) were subject to little error only (the relative standard deviation varied between 3 and 9%). The dependence of the logarithms of the inhibition constants calculated on pH is shown in Fig. 4. It can be observed that constant K_1 depends on pH; the inflection point of the curve obtained lies at a pH close to 6·5. It would thus appear that the association of the first aporheine molecule with the GDH subunit is primarily affected by the net charge of the alkaloid molecule (the pK of aporheine is *c.* 6·3, ref.[10]) even though the presence of other complicating effects cannot be eliminated. It is probable, in accordance with the character of the dependence of log K_1 on pH (Fig. 4), that a positive charge is localized in the neighborhood of the binding site of GDH for the first aporheine molecule; this provides evidence in favor of the conclusions made in ref.[1].

The second binding site of the GDH subunit for aporheine appears only at a pH higher than physiological; the corresponding K_2 constant rapidly decreases with pH and attains a value of *c.* 5 μmol/l at pH about 9 whereas K_1 equals *c.* 60 μmol/l at this pH (Fig. 4). It is likely that this second binding site which binds aporheine

more firmly than the first site, is formed as a result of a conformational change brought about by the binding of the first aporheine molecule at a higher pH. It would appear from the character of the plot of log K_2 *versus* pH (Fig. 4) that some group of the enzyme with a pK around pH 8 participates on the induction of this second binding site. This group with a relatively atypical pK-value among amino acid residues could be most likely Lys 126: according to[4] its amino group has a relatively low pK-value approaching 8.

Even though the described explanation of sigmoidal inhibition of GDH by aporheine at higher pH is most probable in view of the data obtained, it represents only one of the possible alternatives. To obtain a more detailed picture of the mechanism proposed it would be necessary to measure exactly the kinetics of GDH inhibition by aporheine at higher pH-values at various concentrations of the coenzymes and substrates, to examine the binding of aporheine to GDH in the latter media by equilibria techniques other than fluorescence polarization (which yielded relatively inaccurate results[1] with this system), and also to test the effect of aporheine on GDH whose amino acid residues (mainly Lys 126) have been selectively modified.

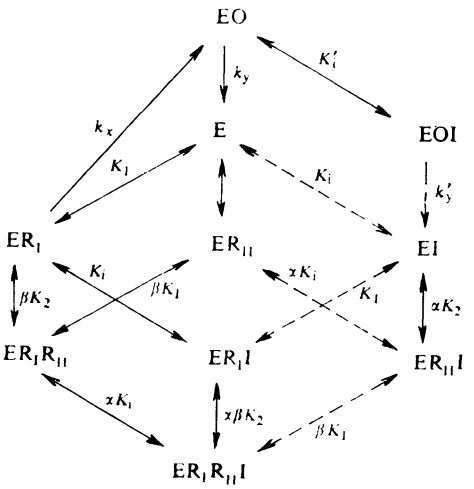
SUPPLEMENT

Analysis of the effects of inhibitors on the kinetics of GDH at high NADH concentrations

The analysis is based on the following assumptions:

1) The complexes of the enzyme with the inhibitors are not catalytically active (the inhibitors are total[1,5]); the binding of NADH to the regulatory site prevents catalytic conversion[11].

2) The inhibitor is either noncompetitive (aporheine[1]) with respect to NADH or purely noncompetitive (thyroxine[5]).



3) Ternary complexes of the inhibitor and NAD are formed[1,5].

4) The kinetics of GDH can be approximated by the rapid equilibrium random mechanism[4] (see below), the decomposition of the enzyme–NAD complex is the step determining the overall reaction rate[4].

5) Since the substrates (*i.e.* α-oxogiutarate and NH_4^+) do not affect the binding of the inhibitors to the enzyme[1,5] they are not considered in the reaction scheme for the sake of simplicity. (Even if they were considered relations similar to those described below would be derived.)

Taking for granted the above assumptions the effect of the inhibitors can be explained by the following scheme (the steps marked by dashed lines occur only with a noncompetitive inhibitor):

In the above scheme the following symbols were used: E enzyme subunit, R NADH, O NAD, I inhibitor, ER_I enzyme–NADH complex (in active center), ER_{II} enzyme–NAD complex (in regulatory site), EO, EI, EOI, ER_IR_{II}, ER_II, $ER_{II}I$, and $ER_IR_{II}I$ corresponding enzyme complexes, K_1, K_2 constants of binding of NADH to the active and regulatory site, K_i, K_i' inhibition constants, α,β-ratios of the corresponding equilibrium constants (α expresses the measure of the effect of NADH in the regulatory site on the affinity of the inhibitor for the enzyme and *vice versa*, β characterizes the effect of the binding of NADH to the regulatory site on the binding of NADH to the active center and *vice versa*), k_x, k_y, k_y' rate constants, $\leftrightarrow$ equilibrium steps, $\rightarrow$ one-way reaction steps.

It can be assumed that the steps described by rate constants k_x, k_y, and k_y' are relatively slow (especially in comparison with the rapid association-dissociation steps described by symbol $\leftrightarrow$). This holds for k_y and k_y' (*cf.* point 4); likewise, the step described by constant k_x is relatively slow since it is a complex process involving the transfer of hydride and the dissociation of the product. Taking these assumptions for granted the above scheme can be solved relatively simply by the method of partial equilibrium according to Cha[6].

The initial reaction rate in the presence of a purely noncompetitive inhibitor is given by:

$$v_0 = E_0 \cdot \frac{k_x R/K_1 \,.\, (k_y + k_y' I/K_i')}{(k_y + k_y' I/K_i')\,(Q + I/K_i + RI/\alpha K_2 K_i) + k_x R/K_1 (1 + I/K_i)},$$

where $Q = 1 + R/K_1 + R/K_2 + R^2/\beta K_1 K_2 + RI/K_1 K_i + R^2 I/\alpha\beta K_1 K_2 K_i$, E_0 is the total enzyme concentration, R the concentration of NADH, and I the concentration of the inhibitor.

Rate v_0 is maximal when $dv_0/dR = 0$. It follows from this relation that the square of NADH concentration at which the reaction rate in the presence of a noncompetitive inhibitor is maximal equals

$$R_m^0 = \beta K_1 K_2 \,.\, (1 + I/K_i)/(1 + I/\alpha K_i)\,.$$

In the absence of the inhibitor $(R_m)_0^2 = \beta K_1 K_2$.

It follows from what has been shown above that the NADH concentration at which v_0 becomes maximal will decrease in the presence of increasing concentrations of an inhibitor purely noncompetitive with respect to NADH (bound to the active center) only if $\alpha < 1$ (*i.e.* if complex $ER_{II}I$ is formed and the presence of NADH in the regulatory site increases the affinity of the inhibitor for the enzyme). If $\alpha = 1$ then $R_m = (R_m)_0$ (the position of the maximum does not change); in the case of labilization ($\alpha > 1$) the maximum will be shifted toward higher values of NADH concentration with the increasing inhibitor concentration.

In case of noncompetitive inhibition with respect to NADH in the active site (*i.e.* if complexes EI and $ER_{II}I$ in the above scheme are not formed and the steps marked by dashed lines cannot be

considered), a slightly simpler equation can be derived for the initial reaction rate:

$$v_0 = E_0 \cdot \frac{k_x k_y R/K_1}{k_y Q + k_x R/K_1(1 + I/K_i)} .$$

The following relation can be obtained for the square of NADH concentration if v_0 is maximal in the presence of a noncompetitive inhibitor:

$$R_m^2 = \beta K_1 K_2 (1 + I/\alpha K_i) ;$$

again $(R_m)_0^2 = \beta K_1 K_2$.

It follows from the relations given above that the maximal value of v_0 will always be shifted toward lower values of NADH concentration in the presence of increasing concentration of an inhibitor noncompetitive with respect to NADH (bound in the active site) if complex ER_1I is formed. If the complex is not formed (and if the inhibitor concentrations are comparable with the K_i-value) the maximum will not be shifted (*i.e.* $R_m \approx (R_m)_0$).

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