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PRODUCT INHIBITION STUDIES OF HUMAN LIVER FORMALDEHYDE DEHYDROGENASE

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Summary

The steady-state kinetic mechanism of human liver formaldehyde dehydrogenase (formaldehyde:NAD^+ oxidoreductase (glutathione-formylating), EC 1.2.1.1) was investigated by product inhibition of the forward and the reverse reactions catalyzed by the enzyme. The results are compatible with a mechanism which contains the random addition to the enzyme of NAD^+ and *S*-hydroxymethylglutathione (the adduct of glutathione and formaldehyde), or NADH and *S*-formylglutathione, and free glutathione as the allosteric activator of the enzyme (Uotila, L. and Mannervik, B. (1979) Biochem. J. 177, 869—878).

Introduction

Formaldehyde dehydrogenase (formaldehyde:NAD^+ oxidoreductase (glutathione-formylating), EC 1.2.1.1) catalyzes the reversible formation of *S*-formylglutathione and NADH from formaldehyde, reduced glutathione and NAD^+ [1]. Formaldehyde and glutathione react rapidly and nonenzymatically to form reversibly a hemimercaptal adduct and a mixture of them thus always contains three species, the uncomplexed forms of formaldehyde (F) and glutathione (G) and the adduct (A). These are related at equilibrium to each other according to the equation

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$$F + G \rightleftharpoons A\ ; \qquad \frac{[F][G]}{[A]} = K_d \tag{1}$$

where K_d is the dissociation constant. A recent steady-state kinetic study of human liver formaldehyde dehydrogenase, involving initial velocity measurements under various conditions [2], gave experimental evidence supporting the earlier suggestion [1,3] that the adduct and NAD^+ are the true substrates of the enzyme. However, another species of the equilibrium mixture of formaldehyde, glutathione and adduct also influenced the rate of the reaction catalyzed by formaldehyde dehydrogenase. The experimental data indicated that free glutathione was essential for the reaction; this compound was suggested to be an allosteric activator of the enzyme [2].

The present report describes further studies of human liver formaldehyde dehydrogenase by product-inhibition experiments. These results support the steady-state kinetic mechanism proposed earlier [2].

Materials and Methods

S-Formylglutathione [4], formaldehyde [1] and human liver formaldehyde dehydrogenase [1] were prepared as earlier described. Other chemicals were purchased and their concentrations determined as described by Uotila and Mannervik [2]. The forward reaction of formaldehyde was measured as before [2]. The equilibrium concentrations of formaldehyde, glutathione and the adduct were calculated by use of the dissociation constant (K_d) of 1.5 mM [1]. The assay mixture for the reverse reaction contained 100 mM sodium phosphate buffer, pH 8.0, varying concentrations of *S*-formylglutathione of NADH and the enzyme. Regression analyses and derivations of rate equations were performed as before [2,5,6]. The concept of generalized inhibition patterns for non-Michaelian kinetics [7] was used.

Results

Product inhibition of the forward reaction. NADH was found to give an inhibition which was generalized competitive with NAD^+ (Fig. 1a). The graphs of the plot were linear at the relatively high constant concentrations of glutathione and formaldehyde in the absence of NADH, but in the presence of NADH, increasing nonlinearity appeared. The inhibition was nonlinear with respect to NADH concentration (Fig. 1b). At a fixed concentration of NAD^+, NADH was generalized noncompetitive with free glutathione when studied at a constant adduct concentration (Fig. 2a). Also in this case the Dixon plot ($1/v$ vs. [NADH]) showed nonlinearities (Fig. 2b). Similar nonlinear noncompetitive inhibition by NADH was obtained with adduct as the variable substrate, when the concentrations of free glutathione and NAD^+ were kept constant. The nonlinearities were less marked than in the graphs of Fig. 2. *S*-Formylglutathione was a generalized noncompetitive inhibitor when the concentration of NAD^+ was varied. It was not possible to test the effect of this product with varying concentrations of adduct or free glutathione because the preparations of *S*-formylglutathione invariably contained some glutathione.

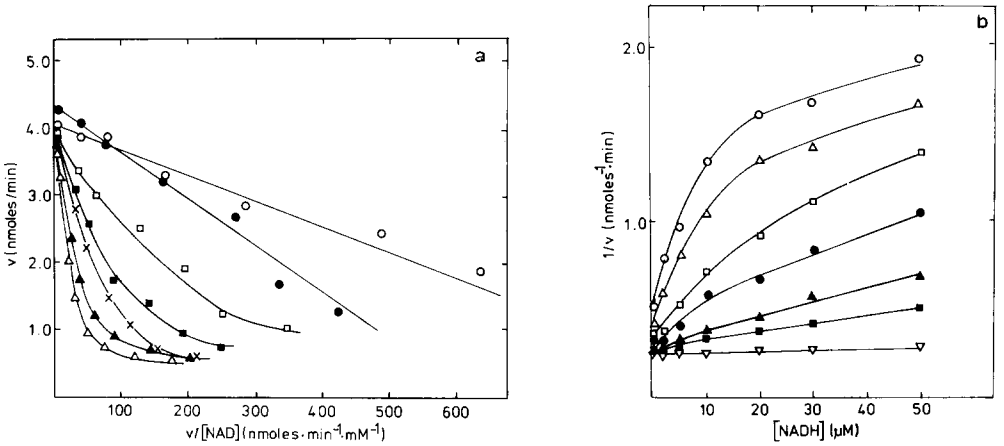

Fig. 1. Product inhibition of the forward reaction by NADH at variable concentrations of NAD^+. The initial velocities were measured as described in the text. (a) v vs. v/[NAD] at NADH concentrations of zero (○), 2 μM (●), 5 μM (□), 10 μM (■), 20 μM (X), 30 μM (▲) and 50 μM (△). (b) $1/v$ vs. [NADH] with NAD^+ concentrations of 3 μM (○), 5 μM (△), 10 μM (□), 20 μM (●), 50 μM (▲), 100 μM (■) and 1 mM (▽). The concentrations of adduct, free formaldehyde and free glutathione were 0.5, 1.5 and 0.5 mM, respectively.

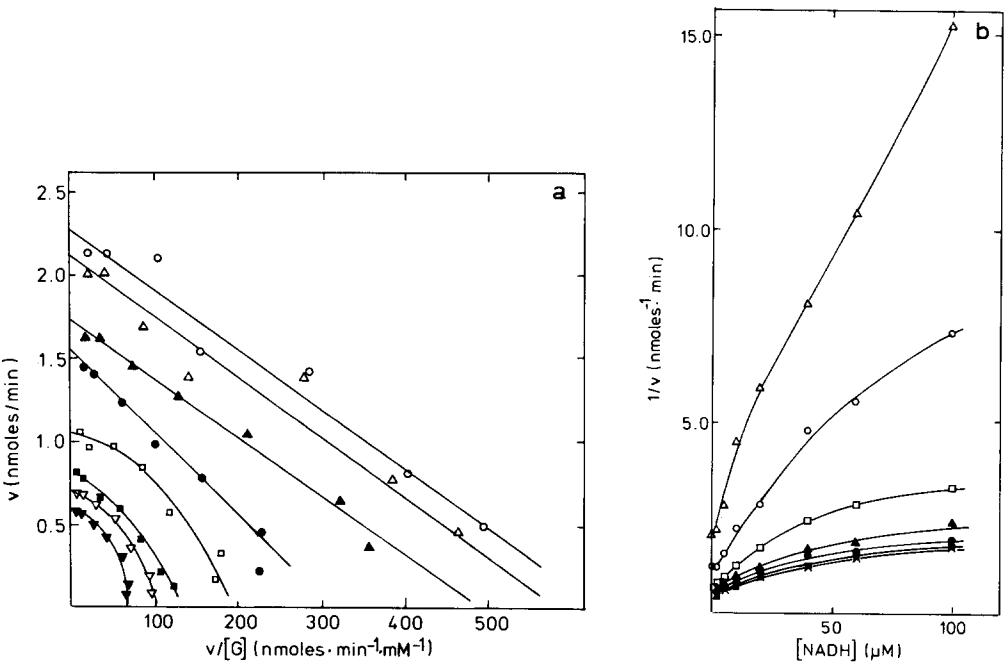

Fig. 2. Product inhibition of the forward reaction by NADH at variable concentrations of free glutathione [G]. (a) v vs. v/[G] at NADH concentrations of zero (○), 2 μM (△), 5 μM (▲), 10 μM (●), 20 μM (□), 40 μM (■), 60 μM (▽) and 100 μM (▼). (b), $1/v$ vs. [NADH] with free glutathione at 1 μM (△), 2 μM (○), 5 μM (□), 10 μM (▲), 20 μM (●), 50 μM (■) and 100 μM (X). The concentrations of NAD^+ and adduct were 30 μM and 20 μM, respectively, throughout the experiment. Formaldehyde concentrations were calculated from Eqn. 1.

Reverse reaction. NAD^+ was a generalized competitive, nonlinear inhibitor when the concentration of NADH was varied at constant *S*-formylglutathione concentration. The results of this experiment were qualitatively similar to those presented for the forward reaction in Fig. 1. When the concentration of *S*-formylglutathione was varied at constant NADH concentration, NAD^+ was a noncompetitive inhibitor. The possibilities for more extensive experiments were limited for the same reason as in the case of the forward reaction.

Discussion

We have earlier presented a random sequential reaction scheme for formaldehyde dehydrogenase, in which the adduct and NAD^+ are substrates. In addition, free glutathione was considered as an essential activator of the enzyme and proposed to bind to an allosteric site [2]. This reaction scheme is presented in Fig. 3. The present product-inhibition data show that NAD^+ and NADH are competing for the same form(s) of the enzyme. The nonlinear dependence of $1/v$ on NADH concentration (Fig. 1b) is consistent with the model of Fig. 3 since four enzyme forms (E, EA, GE, GEA) can bind a molecule of pyridine nucleotide. The alternative reaction paths can explain the nonlinearity. Our earlier experiments with dead-end inhibitors (analogues of NAD^+) have shown that formaldehyde dehydrogenase most probably has no more than one nucleotide-binding site per catalytic subunit [2].

The noncompetitive inhibition by NADH when adduct or free glutathione was varied is also consistent with the mechanism shown in Fig. 3, because neither of these compounds will be bound to the same enzyme forms to which NADH is bound. Both adduct and free glutathione are most probably bound to sites distinct from the nucleotide-binding site. The nonlinearities obtained in the product-inhibition experiments of the reverse reaction suggest that NADH and *S*-formylglutathione are bound to and released from the enzyme in random order.

We have also performed nonlinear regression analyses of our product-inhibition experiments. The rate law for the scheme in Fig. 3 was compared with those of three other schemes discussed earlier (see Scheme 1, A-D, in Ref. 2), as

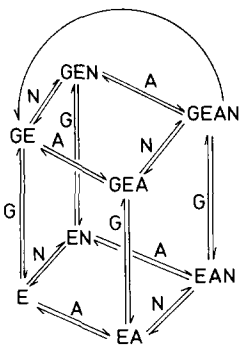

Fig. 3. Reaction scheme for formaldehyde dehydrogenase. A, adduct of formaldehyde and glutathione; E, enzyme; G, free glutathione; N, NAD^+.

well as with several additional models. These comparisons gave objective support for the model of Fig. 3 as the best of the alternatives.

Recently Kato et al. [8] published a steady-state kinetic study on formaldehyde dehydrogenase of *Candida boidinii*, a methanol-utilizing yeast. The enzyme from this yeast has molecular properties and substrate specificity [9] which closely agree with those of the human liver enzyme [1]. The kinetic mechanism suggested by Kato et al., an ordered bi-bi mechanism with NAD^+ as the first substrate, is quite different from that indicated by our results for the human enzyme. The kinetic mechanisms of the human and yeast enzymes may indeed differ, but the study of Kato et al. [8] did not involve sufficiently wide substrate concentration ranges for certain exclusion of nonlinearities (only 3—5-fold variation of substrates according to Figs. 1—3 of Ref. 8, compared to 25—1000-fold variation in the present work). The possible effect of free glutathione on the kinetics was not taken into account by Kato et al. [8]. Competition experiments between adduct, free glutathione and *S*-formylglutathione should give useful additional information on the mechanism of formaldehyde dehydrogenase. However, such experiments have thus far been impossible to carry out, because the unstable *S*-formylglutathione could not be prepared free of glutathione.

Acknowledgement

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