Stopped-Flow Determination of the Active Form of Acetaldehyde in the Liver Alcohol Dehydrogenase Catalyzed Reaction†

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ABSTRACT: A stopped-flow spectrophotometer was modified so that the volumes to be mixed were in a ratio of 1:50. Using this instrument, we have shown that the effective substrate in the reduction of acetaldehyde catalyzed by horse liver alcohol dehydrogenase was the carbonyl form of acetaldehyde and that the enzyme does not catalyze the dehydration of the hydrated form of acetaldehyde. Unlike trifluoroacetaldehyde hydrate, which is a competitive inhibitor with respect to ethanol, acetaldehyde hydrate did not inhibit the enzymatic reaction at concentrations as high as 60 mM.

Carbonyl compounds are capable of hydrating, and aqueous solutions of many aldehydes and ketones are in fact equilibrium mixtures of carbonyl and *gem*-diol species. Although it was initially assumed that the hydrate form was actually the substrate in aldehyde-oxidizing systems (Dixon & Webb, 1964), it has been shown that the carbonyl form of acetaldehyde is the real substrate in systems such as milk or chicken liver xanthine oxidase (Fridovich, 1966), yeast aldehyde dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase (Naylor & Fridovich, 1968), as well as in reducing systems such as yeast alcohol dehydrogenase (Müller-Hill & Wallenfells, 1964). The carbonyl forms of glyoxylic and pyruvic acids also are the effective substrates with tartronate–semialdehyde synthase (Hall & Kezdy, 1970) and lactate dehydrogenase (Tienhaara & Meany, 1973), respectively. In this latter case, however, it has been shown that the hydrated pyruvate accounts for significant "substrate inhibition".

In the dismutase reaction catalyzed by liver alcohol dehydrogenase, the K_m of acetaldehyde is 100 mM (Dalziel & Dickinson, 1965), suggesting that the hydrated form of acetaldehyde has no great affinity for the complex enzyme–NAD^+. In the present study, however, we found that trifluoroacetaldehyde is an efficient inhibitor, competing with the reduced substrate ethanol. Since trifluoroacetaldehyde is highly hydrated in aqueous solution (Guthrie, 1975), the inhibition raised the question of the role of the hydrate of acetaldehyde. This question is particularly pertinent to discussions of the pre-steady-state kinetics of horse liver alcohol dehydrogenase (Baici & Luisi, 1977; Kvassmann & Pettersson, 1976).

From the three-dimensional structure of alcohol dehydrogenase, it has been suggested that the zinc atom present in the active site plays the role of a Lewis acid catalyst (Bränden et al., 1975). This metal could also catalyze the dehydration of acetaldehyde hydrate, so that acetaldehyde in the carbonyl form and its hydrate would both be substrates. This possibility could only be ruled out by comparing the reduction rate measured with anhydrous acetaldehyde to the reduction rate measured with the hydrate equilibrium mixture.

Since the hydration of carbonyl compounds and especially acetaldehyde is quite a fast process and exhibits general acid–base catalysis (half-times ranging from 0.3 to 60 s; Bell et al., 1956) and the approximately 1:1 equilibrium is reached in less than 3 min (Bell, 1966), accurate measurements of the rate of reduction of the unhydrated species require that the hydration be negligible during the period of measurement.

In this paper, we show that accurate steady-state kinetic measurements of the rate of reduction of acetaldehyde can be performed on the anhydrous form of acetaldehyde in aqueous media within 0.5 s.

Experimental Section

Material and Methods

Horse liver dehydrogenase was from Boehringer (Mannheim, West Germany). It was dialyzed 48 h against 20 mM phosphate buffer, pH 7.5, before use. NADH was from Boehringer. Tetramethylurea was from Fluka (Buchs, Switzerland). It was distilled four times under reduced pressure on a Fischer Spaltrohr system (Fischer, 53-Bonn, Bad Godesberg 1) before use. Acetaldehyde was prepared from paraldehyde (Prolabo, Paris, France) and distilled four times under argon before use, the last distillation being performed not more than 6 h before the kinetic determination. Trifluoroacetaldehyde was prepared from its methyl hemiacetal (Fluka) (5 g) by treatment with a mixture of phosphorus pentoxide (2.2 g) and sulfuric acid (18 g) under argon. Trifluoroacetaldehyde was condensed at −78 °C (2.6 g) and treated with water (0.5 mL). The trifluoroacetaldehyde hydrate was then distilled (1.5 g; bp 105–106 °C; lit. 105 °C; Husted & Ahlbrecht, 1952), and the structure was checked by NMR and was found to be in agreement with published data (Guthrie, 1975). Dimethoxyethane was from Fluka. It was refluxed on lithium aluminum hydride for 3 h, distilled, and used immediately.

K_i measurements of trifluoroacetaldehyde hydrate and tetramethylurea were performed in 0.1 M phosphate buffer, pH 7.5, at a saturating concentration of coenzyme: 10^{-4} M for NADH (acetaldehyde concentration varying between 3 × 10^{-4} and 0.5 × 10^{-4} M) and 5 × 10^{-4} M for NAD^+ (ethanol concentration varying between 3 mM and 0.5 mM).

Solutions, 1 M, of acetaldehyde in tetramethylurea were prepared by weighing an appropriate amount of anhydrous acetaldehyde in a 5-mL round-bottomed flask fitted with a serum cap and by adding tetramethylurea using a syringe. Appropriate amounts of this solution were diluted with tetramethylurea (or phosphate buffer) similarly to yield acetaldehyde solutions of differing concentrations, which were calculated using the weight ratios. The solutions were used within 4 h.

Stopped-flow measurements were carried out with a Durrum Model D 130 stopped-flow spectrophotometer equipped with a Kel-F valve block. A mixing ratio of 1:50 was used. The

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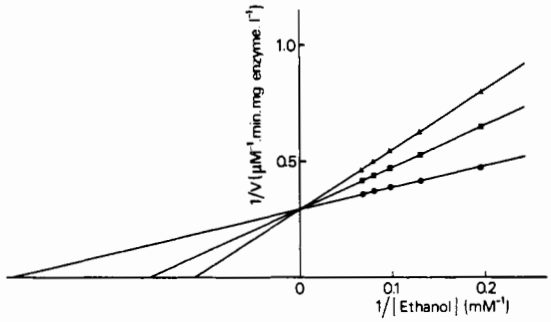

FIGURE 1: Inhibition of ethanol oxidation by trifluoroacetaldehyde hydrate. Concentration of trifluoroacetaldehyde: (●) none; (■) 0.2 mM; (▲) 0.4 mM. Velocities are expressed as micromoles of NADH formed per milligram of enzyme per minute.

driving syringes were 5 mL and 0.1-mL gas-tight Hamilton models. The stopping syringe volume was adjusted to 2 mL. The measurement cell had a 2-mm light path. Great care was taken with the tightness of valves, syringes, and other joints.

Data of absorbance vs. time were acquired with a Tektronix Model 5103 storage oscilloscope and a Datalab DL 905 transient recorder. The stored signal was plotted on a Tacussel Model EPL 1 recorder.

For stopped-flow experiments with anhydrous acetaldehyde, the small syringe contained the acetaldehyde solutions in tetramethylurea. The large syringe contained a solution prepared as follows: to 49 mL of a 1.58×10^{-4} M NADH and 1.67×10^{-6} M LADH in phosphate buffer (20 mM, pH 7.5) was added 1 mL of twice distilled water. For experiments with hydrated acetaldehyde, the small syringe contained the aldehyde solutions in phosphate buffer (20 mM, pH 7.5). The large syringe contained a solution prepared as follows: to 49 mL of a 1.58×10^{-4} M NADH and 1.67×10^{-6} M LADH in phosphate buffer (20 mM, pH 7.5) was added 1 mL of tetramethylurea.

Procedure

(a) The pistons of the drive syringes were removed from the corresponding holder, and the block channel between the reservoir and the drive syringes was rinsed with twice distilled water and then dried with purified air.

(b) The channel and the drive syringe were then filled with aldehyde solution from the reservoir syringe, after which the piston of the drive syringe was mounted.

(c) Immediately prior to the measurement, the block channel was drained from the reservoir syringe to the cuvette.

In these conditions, the mixing time determined using a solution of methylene blue in tetramethylurea for the small syringe was smaller than 10 ms, and the reproducibility was better than 2%. The temperature was maintained to 22 °C, and the spectral measurements were performed at 340 nm. The total recording time was 0.5 s.

Results and Discussion

The occurrence of trifluoroacetaldehyde as its hydrate in aqueous solutions (Guthrie, 1975) may explain the total lack of its enzymic reduction by NADH. Trifluoroacetaldehyde hydrate was found to be a noncompetitive inhibitor with respect to acetaldehyde (K_i = 0.15 mM) and a competitive inhibitor with respect to ethanol (K_i = 0.2 mM) with horse liver alcohol dehydrogenase (Figure 1). This suggested the formation of a ternary complex: enzyme–NAD^+–trifluoroacetaldehyde hydrate. In view of this result, the role of the hydrate of acetaldehyde in the mechanism of alcohol dehydrogenase was raised.

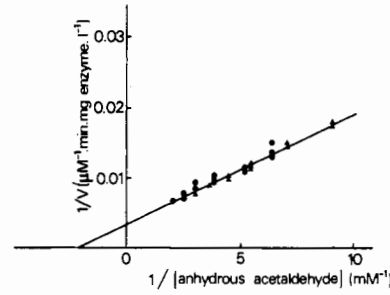

FIGURE 2: Rate of reduction of anhydrous (●) and hydrated equilibrium mixture (▲) of acetaldehyde. Concentration of the acetaldehyde is expressed as [anhydrous form] (mM^{-1}). Velocities are expressed as micromoles of NADH disappearing per milligram of enzyme per minute. The factor used to correct the total acetaldehyde concentration for its hydrated form was 0.45 derived from the ratio [hydrate]/[carbonyl] = 1.22 which was directly determined.[2]

The rate of hydration of acetaldehyde is quite high and the equilibrium in water is reached in less than 3 min (half-times ranging from 0.3 to 60 s; Bell et al., 1956; Bell, 1966). A general acid and base catalysis has been described for this reaction (Pocker & Meany, 1967; Lienhard & Anderson, 1967). The time scale excluded the usual spectrophotometric measurements. In the classical stopped-flow instrument, the volumes to be mixed are in the 1:1 ratio. For the reduction of the carbonyl form of acetaldehyde, the solution had to be anhydrous and the use of large amounts of solvent would have interfered with the enzymatic reaction. Therefore in order to study this reaction in conditions close to those used in classical kinetic studies, it seemed more appropriate to modify the stopped-flow instrument and to use a 1:50 ratio for the volumes to be mixed. The efficient mixing of the solutions occurred within 5 ms. This was proved using colored solution in tetramethylurea for the small syringe and water for the large one.[1] Tetramethylurea was chosen as solvent for anhydrous acetaldehyde for two reasons. It is inert to acetaldehyde and was also found to be a very poor inhibitor with liver alcohol dehydrogenase (K_i = 0.6 M) with respect to acetaldehyde. Two-hour incubation of the enzyme with tetramethylurea at the concentration used here did not alter the enzymic activity. Dimethoxyethane (Müller-Hill & Wallenfells, 1964) proved to be a stronger inhibitor and was disregarded for further experiments.

In a first set of experiments, the small syringe contained the anhydrous acetaldehyde solution in tetramethylurea, while the large syringe contained the enzyme and NADH in 20 mM buffer. In a second set of experiments, aqueous solutions of acetaldehyde were present in the small syringe, whereas the large one contained enzyme, NADH, and tetramethylurea in 20 mM buffer. After a mixing time of 5 ms, a linear decrease of NADH concentration during the next 50 ms was observed. The observed rates are presented in the Lineweaver–Burk plot (Figure 2), using for acetaldehyde concentration that of the carbonyl form.[2] In these conditions no effect of the hydrated

[1] The reproducibility of our results justified a posteriori this modification (see Figure 2); each point (● or ▲) corresponds to a series of three successive reproducible measurements.

[2] Kinetic measurements showed that within 0.5 s (the duration of each measurement) no noticeable decrease occurred in optical density at 278 nm when a 1.0 M solution of anhydrous acetaldehyde in tetramethylurea was mixed with the aqueous buffer used in all the experiments. At this wavelength, the half-time of the hydration of acetaldehyde in 20 mM phosphate buffer, pH 7.5, was found to be 9.0 ± 0.3 s and a ratio [hydrate]/[carbonyl] = 1.22 was determined (lit. 1.2; Bell, 1966; Kurz, 1967).

form of acetaldehyde was observed at a concentration as high as 0.4 mM.

These experiments proved that, for liver alcohol dehydrogenase, the carbonyl form is indeed the active substrate and that the catalysis of the dehydration of the hydrated form of acetaldehyde by the enzyme is not observable. This agrees with the general picture which emerges from the studies of carbonyl reducing enzymes. On the other hand, the hydrated form of acetaldehyde did not influence the kinetics of reduction of the carbonyl form, excluding the formation of strong complexes of the type enzyme–coenzyme–hydrated form. This is in contrast with the behavior of the hydrated form of trifluoroacetaldehyde which is a competitive inhibitor with respect to ethanol and thus binds to the enzyme–NAD^+ complex. Since no inhibition for the acetaldehyde hydrate has been detected, the trifluoromethyl group must tighten the binding. Such behavior is reminiscent of the increased binding observed on replacement of an acetyl by a trifluoroacetyl group (Renaud et al., 1978).

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Affinity Labeling of an Allosteric GTP Site of Bovine Liver Glutamate Dehydrogenase by 5′-*p*-Fluorosulfonylbenzoylguanosine†

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ABSTRACT: In the presence of DPNH, native glutamate dehydrogenase binds, with markedly different affinities, 2 mol of the allosteric inhibitor GTP per peptide chain. In contrast, only 1 mol of GTP is bound in the absence of coenzyme. Incubation of enzyme with the guanosine nucleotide analogue 5′-*p*-fluorosulfonylbenzoylguanosine does not affect the intrinsic catalytic activity of the enzyme as measured in the absence of regulatory compounds, but leads to a progressive decrease in the sensitivity to inhibition by GTP. The modified enzyme binds only 1 mol of GTP per peptide chain, in the presence or absence of DPNH, implying that reaction with 5′-*p*-fluorosulfonylbenzoylguanosine eliminates one of the allosteric sites for GTP. In contrast, the Michaelis constants for substrates and the ability of the enzyme to be inhibited by high concentrations of DPNH are not appreciably changed by the modification reaction. Although the affinity for the activator ADP is not altered, the maximum extent of activation is decreased. The rate constant for reaction of glutamate dehydrogenase with 5′-*p*-fluorosulfonylbenzoylguanosine has been measured from the time dependence of the decreased inhibition by 1.1 μM GTP; this rate constant is specifically and strikingly decreased by low concentrations of GTP in the presence of reduced coenzyme but not by substrates, DPNH alone or ADP either with or without DPNH. The extent of covalent incorporation of radioactive 5′-sulfonylbenzoylguanosine is directly proportional to the percent decrease in GTP inhibition, a maximum alteration in sensitivity to GTP being observed when approximately 2 mol of 5′-sulfonylbenzoylguanosine is incorporated per enzyme subunit. Only 1 mol of reagent per peptide chain is covalently bound in the presence of GTP and reduced coenzyme, which protect the enzyme against the decreased response to GTP but do not prevent the decreased activation by ADP. In contrast, about 2 mol of reagent per enzyme subunit is incorporated in the presence of ADP and DPNH, which protect neither against the reduced response to ADP nor to GTP. These results suggest that incorporation of 1 mol of 5′-*p*-sulfonylbenzoylguanosine specifically causes elimination of one of the GTP sites of the native enzyme and that this change is responsible for the decreased sensitivity to GTP inhibition. Incorporation of the second mole of 5′-*p*-sulfonylbenzoylguanosine may occur at a site distinct from the recognized allosteric sites and indirectly cause a change in the extent of activation by ADP.

Bovine liver glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) is an allosteric enzyme the activity of which is influenced by a variety of compounds which bind to distinguishable but mutually interacting sites. Notable among these compounds are GTP which inhibits, ADP which activates, and DPNH which, at relatively high concentrations, inhibits by binding to a site distinct from the catalytic site (Goldin & Frieden,

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