Proton NMR measurements of hydrogen exchange at the C-3 position of 3-hydroxybutyrate in suspensions of rat liver mitochondria

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Rat liver mitochondria suspended in buffer made with ^2H_2O catalyse exchange of the C-3 proton of added D-3-hydroxybutyrate with solvent deuterons. The kinetics of this process can be followed using spin-echo proton NMR. The observed isotope exchange velocity is sensitive to the activity of D-3-hydroxybutyrate dehydrogenase, which is an integral protein of the inner mitochondrial membrane. A method is described which can be used to obtain the specific isotope exchange velocity of the enzyme in the intact mitochondrion.

Proton NMR *Isotope exchange* *Mitochondria* *3-Hydroxybutyrate*

1. INTRODUCTION

Proton NMR measurements of enzyme-catalyzed isotope exchange have proved to be useful for studying enzyme kinetics in intact cells [1–4]. The strategy employed in these studies has been to compare the exchange kinetics displayed by a specific enzyme in the cell with the properties it expresses in a reconstituted in vitro system. In this way the nature of the intracellular environment can be investigated. If the observed exchange reaction is composed of a series of coupled reactions, each catalyzed by a different enzyme, then the contribution of a specific enzyme may be determined as in [4]. The method involves titrating the enzyme's activity in the cell with a specific irreversible inhibitor and then measuring the dependence of the overall exchange velocity on the spectrophotometrically assayed activity of the enzyme in cell extracts. We here show that the same technique can be used to determine the activity expressed by D-3-hydroxybutyrate dehydrogenase (BOHDH) in intact rat liver mitochondria.

2. MATERIALS AND METHODS

Rat liver mitochondria were isolated in 200 mM mannitol/70 mM sucrose/2 mM EGTA/0.5% (w/w) BSA/10 mM HEPES (pH 7.0) as in [5]. Mitochondria were washed twice in isolation medium containing no BSA. To exchange solvent, they were subsequently washed in the same buffer made with ^2H_2O. The protein content of the suspension was determined by means of the Biuret method [6] which was calibrated using BSA. A typical suspension used in the experiments described contained about 50 mg/ml mitochondrial protein. Spin-echo NMR spectra were recorded at 468 MHz as in [3] using a 90°-τ–180°-τ pulse sequence with a τ-value of 68 ms. The sample temperature was 22°C. Reactions were initiated by

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Abbreviations: BOH, 3-hydroxybutyrate; BOHDH, 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30); HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis-[β-amino ethyl ether]-*N,N'*-tetraacetic acid; v_{obs}, observed isotope exchange velocity for the exchange system

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adding 10 μl of 0.5 M DL-3-hydroxybutyrate (BOH), pH 7.0, to 0.49 ml mitochondrial suspension. The mitochondria were not oxygenated during the NMR experiments. The equilibrium isotope exchange velocities were obtained by means of a least squares fit of peak intensities to an exponential decay function.

Assays for BOHDH activity were performed as in [7]. The mitochondria were disrupted by ultrasonic vibration before assay of enzyme activity.

3. RESULTS AND DISCUSSION

Mitochondria have been shown to catalyze exchange of the B face C-4 hydrogen of the nicotinamide ring of NADH with solvent [8]. Solvent label can then exchange between the coenzyme and the C-3 position of D-3-hydroxybutyrate in the reaction catalyzed by D-3-hydroxybutyrate dehydrogenase. This enzyme is specific for the D-isomer of 3-hydroxybutyrate and is found in relatively high activity in liver mitochondria of various species [9]. The enzyme from *Rhodopseudomonas sphaeroides* has been shown to have B face stereospecificity for the coenzyme [10].

Addition of DL-BOH to rat liver mitochondria suspended in a buffer made with ^2H_2O results in exchange of the C-3 proton of the D-form. This exchange can be followed in spin-echo spectra by observing changes in the phase modulation of the coupled C-4 methyl resonance. The modulation is of the form $\cos[2\pi J\tau]$ [11]. Thus, after a delay time $\tau = 1/2\,J$ the methyl resonance of the fully protonated BOH is inverted. If the C-3 proton is exchanged for a solvent deuteron, phase modulation of the C-4 methyl resonance due to homonuclear coupling is removed. Changes in the amplitude of the methyl resonance are correlated, therefore, with exchange of the C-3 proton. By observing inversion of the signal from the 3 protons of the methyl group, the effect of the exchange of the single proton at the C-3 position is amplified. The C-3 proton multiplet can be observed directly, but this is difficult due to its close proximity to the solvent and buffer resonances.

Full inversion of the methyl resonance is not observed since the enzyme exchanges only the D-isomer. The L-isomer remains protonated at the C-3 position and thus the methyl resonance of this species retains an inverted phase. Since the two isomers are present in nearly equimolar amounts isotope exchange results in decay of the initially observed methyl resonance to about the baseline.

In cases where isotope exchange proceeds through a linear series of coupled reactions, the inverse of the observed overall equilibrium isotope exchange velocity v_{obs}, defined as the isotope exchange velocity under conditions where the substrates are at chemical equilibrium [12], is equal to the sum of the reciprocals of the velocities v_i of the individual steps [13]:

$$1/v_{obs} = \sum_i 1/v_i \qquad (1)$$

This relationship originally derived for trace isotope exchange, also applies to bulk isotope exchange [3]. The exchange steps may also involve non-enzymatic reactions or processes such as membrane transport.

The isotope exchange velocity of an individual enzyme can be obtained using a technique which was used to measure the activity expressed by glyceraldehydephosphate dehydrogenase in the intact human erythrocyte [4]. The method involves titrating the activity of the enzyme with a specific irreversible inhibitor. The observed overall exchange velocity shows the following dependence on the activity of the enzyme:

$$1/v_{obs} = 1/v_r + 1/\alpha A \qquad (2)$$

where:

A is the spectrophotometrically assayed activity of the enzyme in a tissue extract;

$1/v_r$ is the sum of the reciprocals of the velocities of the other exchange processes;

α is the specific isotope exchange velocity of the enzyme expressed as μmol substrate exchanged $\cdot$ min^{-1} $\cdot$ unit of enzyme activity^{-1}.

Thus a plot of $1/v_{obs}$ *vs* $1/A$ yields α and v_r.

The activity of BOHDH can be titrated in intact mitochondria using *N*-ethylmaleimide (NEM), a potent inhibitor of the enzyme [14] which is a relatively specific inhibitor of the enzyme in mitochondria [15]. The effect of adding varying concentrations of NEM to a mitochondrial suspension on the observed exchange of BOH is shown in fig.1. Immediately after the NMR experiment the BOHDH activity in sonicated extracts was measured spectrophotometrically in a standard

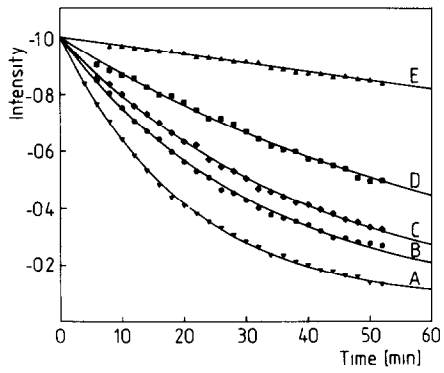

Fig.1. Plots of BOH methyl peak intensity *vs* time following the addition of BOH to a mitochondrial suspension. Inhibition of the exchange by NEM. Mitochondria were suspended in the isolation medium made with ^2H_2O as described in section 2. Spectra were accumulated every 2 min (80 scans) following the addition of 10 mM DL-BOH to a mitochondrial suspension which contained the following concentrations of NEM: (a) 0.0 μM; (b) 96 μM; (c) 145 μM; (d) 193 μM; (e) 241 μM. The lines drawn through the points represent least squares fits to an exponential decay function.

assay system (see section 2). The assayed activity showed a similar decline to the decrease in exchange velocities. From a plot of $1/v_{obs}$ *vs* 1/BOHDH activity (fig.2) a specific isotope exchange velocity for the enzyme of 0.11 μmol $\cdot$ min^{-1} $\cdot$ unit^{-1} of enzyme was obtained.

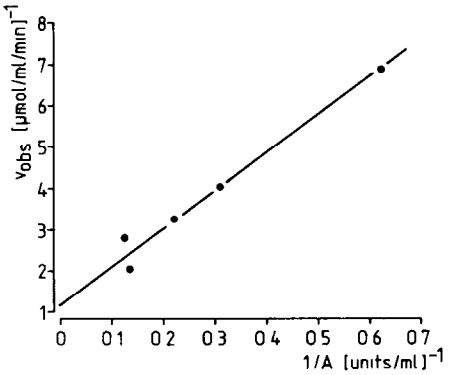

Fig.2. Plot of $1/v_{obs}$ *vs* 1/BOHDH activity. Exchange measurements were performed as described in section 2 and in the legend to fig.1. The BOHDH activity was measured spectrophotometrically in mitochondrial sonicates following completion of the exchange measurements.

This value allows calculation of a sensitivity coefficient, Z, a parameter defined in [16] as:

$$Z = \frac{\delta F/F}{\delta E/E} \quad (3)$$

where:
F is pathway flux;
E is the catalytic activity of any one enzyme.

The sensitivity coefficient for BOHDH, with respect to solvent exchange of the C-3 proton of BOH, was calculated to be about 0.7. The exchange is, therefore, relatively sensitive to the activity of this enzyme.

Although the technique has yielded a value for the specific exchange velocity of the enzyme a number of factors must be considered in assessing the validity and significance of this result.

Transport of BOH across the mitochondrial membrane is expected to be fast relative to the exchange velocity [17]. There is no indication of an inhomogeneous distribution of BOH between the cytoplasm and mitochondria in rat liver [18]. The intra- and extramitochondrial concentrations should, therefore, rapidly reach equilibrium on the time scale of the exchange measurements. The existence of near equilibrium concentrations of the mitochondrial substrates involved in the exchange is indicated by the following observations. There is no change in BOH concentration during the exchange measurements, added acetoacetate is in part reduced to BOH suggesting a largely reduced redox chain and incubation of the mitochondrial suspension for 1–3 h before the NMR experiment had no effect on the subsequently measured exchange velocity. Although NEM appears to be relatively specific in its inhibition of BOHDH in mitochondria [15] it is known to inhibit mitochondrial phosphate transport [19]. The effect of this and other possible effects of the reagent on the observed exchange velocity have not yet been assessed although they are not expected to be significant.

The BOH exchange pathway is not known in detail, however equations 1 and 2 will be valid for BOHDH since this enzyme is a terminal enzyme in the pathway and there are no parallel pathways which can bypass this step. This is demonstrated by the fact that complete inhibition of the exchange is obtained at high concentrations of NEM. The involvement of parallel pathways in the ex-

change of label between NADH and solvent is indicated by the effect of rotenone on the exchange. Addition of 20 μM rotenone resulted in a 55% inhibition of the exchange. Higher concentrations of rotenone cause no further increase in the inhibition. If the rotenone-sensitive step is completely inhibited then this indicates that there must be a parallel exchange pathway.

In conclusion, we have shown that the isotope exchange technique can be used to determine the activity expressed by BOHDH in the intact mitochondrion. Isolation of the enzyme [20] and studies of its exchange properties in an in vitro system should permit investigation of its mitochondrial environment. This is particularly interesting in view of the dependence of the enzyme's activity on its lipid environment [21]. Further work with selective inhibitors should allow determination of the quantitative contribution of some of the other enzymes involved in the exchange and investigation of their kinetic properties.

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