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Oscillating enzyme-bound NADH in glycolysis

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In glycolyzing cell-free cytoplasmic medium extracted from yeast *Saccharomyces cerevisiae*, the action spectrum of oscillation has an absorption maximum around 335 nm, nearly coinciding with that of the yeast alcohol dehydrogenase (ADH)-NADH complex due to its bound NADH. Our approximate calculations based on the amount of this enzyme and coenzyme NADH present in the extract suggest that the ADH-NADH complex alone can account for 90% or more of the total absorbance change.

Glycolysis; Oscillation; NADH, enzyme-bound; Action spectrum; Dialysis

1. INTRODUCTION

Glycolytic oscillation observed in cell-free cytoplasmic medium extracted from the yeast *Saccharomyces cerevisiae* has been extensively studied and is the best understood oscillator of a multienzyme system [1–3]. It serves as an example of nonlinear behaviour in metabolism. A thorough understanding of its mechanistic details is of general significance, especially for cell physiology.

Oscillations are readily induced in the yeast extract by a single addition of trehalose or continuous infusion of glucose. The most convenient ways of monitoring the oscillation are the continuous recording of fluorescence emission (excitation at 360–366 nm, emission at 450–460 nm) and the absorbance between 300 and 380 nm. However, the identity of the oscillating species as detected by fluorescence or absorbance remains open. It is generally assumed to be a protein-bound NADH and has been suggested as being NADH bound to alcohol dehydrogenase (ADH) [3,4].

Here, we report our recent work on the nature of the protein-bound NADH and conclude that the yeast ADH-NADH complex with an absorption peak at 335 nm is indeed responsible to a considerable extent for the absorbance oscillation in the cell-free extract.

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2. MATERIALS AND METHODS

All experiments were performed with a cell-free extract prepared from commercial baker's yeast (*S. cerevisiae*) according to [5]. The protein content of the extract was 77 mg/ml unless specified otherwise.

Either a Varian Cary-219 or a Perkin-Elmer Lambda 9 UV/VIS/NIR spectrophotometer was used for absorption measurements. HPLC apparatus (Beckman) and a Shimadzu Chromatopac C-R2A integrator were applied for quantitative analysis of NAD and NADH coenzymes.

The total amounts of NAD and NADH were estimated by HPLC after removing the proteins firstly by treatment with acid (2 M $HClO_4$, equal volumes) or by heating (boiling water, 5 min) and then centrifugation of the extract. The value of total NADH thus obtained should be considered as the lower limit because the acid/heat treatment of the extract leads to partial destruction of NADH [6]. The amounts of free NAD and NADH were estimated by dialysis (Visking membrane) of the extract against buffer (10 mM phosphate, pH 6.5) at room temperature with magnetic stirring for at least 2 h. The concentration of free NADH was greatest after 2 h dialysis. This decreased by 30% after 4 h and by 50% after 24 h due to oxidation and enzymic degradation. The value of free NADH reported here is considered to be a good estimate of the upper limit, the true value possibly being at most 20% higher.

In all cases, oscillations were induced by a single addition of trehalose (81 mM) and potassium phosphate (42 mM) and were monitored at room temperature.

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3. RESULTS AND DISCUSSION

A direct way of identifying the oscillating species in such multienzyme systems is the determination of its action spectrum which is defined here as the wavelength dependence of the amplitude of oscillation. This definition is comparable to that given in [3].

A typical example of a difference spectrum recorded for an oscillating sample of the extract against a non-oscillating one is given in fig.1. The curves comprise 10 scans with an interval of 2.5 min between scans (for labelling see legend to fig.1). A representative action spectrum derived from these difference spectra is shown in fig.2 (trace A). The maximum absorbance varies slightly between individual measurements with the mean peak being at 333 nm.

Alternatively, we determined the action spectrum by monitoring the time course of oscillation at 8 preselected wavelengths between 300 and 380 nm and recorded the corresponding amplitude of the oscillation as depicted by trace B in fig.2,

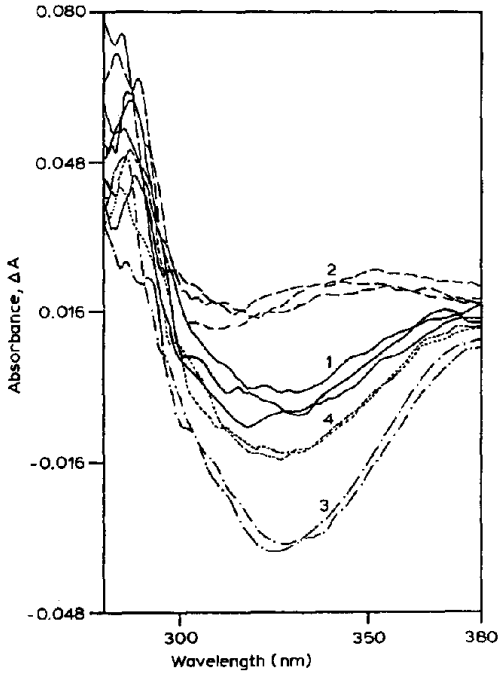

Fig.1. Difference absorption spectra of an oscillating sample of yeast extract vs a non-oscillating sample. A 1 mm quartz cuvette was used. Time sequence, 1⟶2⟶3⟶4; scan rate, 4 nm/s; period of oscillation, 12 min.

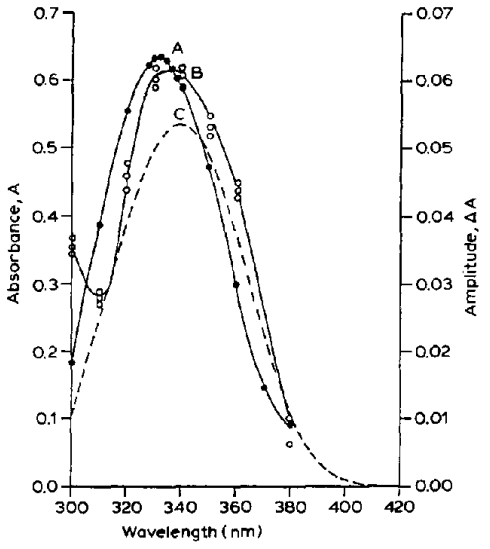

Fig.2. (A,B) Action spectrum for oscillation obtained using two different methods (see text). (C) Absorption spectrum of 1 mM NADH in buffer (10 mM phosphate, pH 6.5).

with the maximum for a series of experiments being within the same range as that determined from the results of the analysis (trace A). Both spectra show the same half-width.

For comparison, fig.2 (trace C) represents the absorption spectrum of NADH in solution. The half-width of all three curves is almost identical. The absorption peaks of curves A and B are clearly shifted toward the blue relative to that of free NADH.

Comparison of the blue-shifted absorption peak of the action spectrum with the absorption maxima in the spectra of protein-bound NADH reported in [7] strongly suggests its identification as the ADH-NADH complex (blue shift, 5 nm).

Since participation of free NADH in oscillating glycolysis of the cell-free extract cannot be evaluated on the basis of the action spectrum, which gives no indication of a prominent component with the maximum at 340 nm, we employed HPLC to assess the possible contributions from the free species.

The equilibrium distribution of bound and free NAD and NADH in the extract is listed in table 1 and compared with the published data. The total amount of NAD agrees well with the values reported in [3], and the free form of NAD ac-

Table I

Distribution of bound and free NAD and NADH in a yeast extract (50 mg/ml protein)

Species	Concentration (μM)	Literature data [3] (μM)
Total NAD	800–1000	1000
Free NAD	200	–
Total NADH	$\geq$ 50–70[a]	300
Free NADH	$\leq$ 10–20[a]	–

[a] $n = 5$

counts for about 20% of the total. Comparing the results of the analysis with data in the literature [3], the free form of NADH accounts for less than 10% of the total. The amplitude of oscillation in the absorbance amounts to a value of up to 0.07 at 340 nm in a 1 mm cuvette (see fig.2), which converts to a concentration change of 120 μM ($\epsilon_{340} = 6 \times 10^3\ M^{-1} \cdot cm^{-1}$). This is considerably greater than the free NADH concentration. Therefore, free NADH can only account for a small part of the absorbance change and the contribution by free NAD is even smaller due to its very low absorption.

On the other hand, there are NAD and NADH-requiring dehydrogenases other than ADH in the extract. The concentration changes in NAD and NADH induced by the glycolytic flux necessarily result in dissociation and association of the coenzymes from and to these enzymes. In principle, all of these changes could contribute to the optical recordings. However, no evidence exists for a major contribution from NAD-enzyme complexes during oscillation because $\Delta[NADH]_{total}$ agrees with ΔA within around 10% (Boiteux, A., personal communication). Due to other sources of error, the actual contribution from all NAD-enzyme complexes is no more than several percent. As pointed out above, the absorption maximum of the yeast ADH-NADH complex matches best that of the action spectrum. Both the NAD and NADH complexes of GAPDH show absorption maxima at wavelengths more than 10 nm longer than 340 nm [7,8]. Other NADH-binding yeast enzymes may be considered as potential candidates only if their bound NADH also shows a similar blue shift. However, no such data are available in the literature.

The maximum activity of ADH in the extract assayed using the standard method [9] corresponds to a concentration of 30–40 μM; such a procedure for estimating enzyme content in the yeast extract appears justified in view of a previous work on highly concentrated enzyme solutions [10]. This means that ADH can bind up to 120–160 μM NADH. Therefore, the concentration oscillation of NADH bound to ADH alone can account for the major part of ΔA and consequently an even larger proportion of the change due to all enzyme-bound NADH.

Based on all available experimental data, we conclude that the yeast ADH-NADH complex is responsible for the absorbance change and can be considered to be the oscillating species which we record. Furthermore, the oscillation in fluorescence emission and absorbance should be in phase, since the NADH bound to ADH has an increased fluorescence yield. This was in fact observed in our laboratory.

Thus, the availability of NADH coenzyme to ADH should be one of the factors leading to the oscillatory ADH activity, a property which has not been emphasized sufficiently. Computer simulations on the basis of previous models [11,12] are in progress in this laboratory to explore this dynamic feature.

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