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NAD(P)$^+$ REDOX COMPARTMENTATION IN EHRLICH ASCITES TUMOUR CELLS

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SUMMARY

1. Normoxia–anoxia transitions and use of glycolytic inhibitors and substrates have made possible a distinction between NAD(P)H redox changes in the mitochondrial and cytosolic compartments of ELD (Ehrlich-Lettré hyperdiploid) ascites tumour cells.

2. Fluorescence (F) and absorbance (A) of NAD(P)H have been measured simultaneously in whole-cell suspensions. The F/A ratios calculated are higher for mitochondrial than cytosolic nicotinamide nucleotides.

3. A difference has also been observed between the absorption maxima of NAD(P)H in the two compartments. Such maxima are at lower wavelengths for mitochondrial nicotinamide nucleotides.

4. These results show that a tool has been obtained for distinguishing different species of nicotinamide–adenine dinucleotides within the intact cell by the relative quantum efficiency of the fluorescence and the peak position of the absorption spectra. Furthermore, indications of interactions between the cytosolic and mitochondrial pools of nicotinamide nucleotides in ELD ascites tumour cells are presented.

INTRODUCTION

Previous work[1], concerned with the analysis of the state of nicotinamide–adenine dinucleotides in intact cells, has allowed us to conclude that, depending on the cell type, some fluorescence emission characteristics (peak position and intensity ratio at 465 and 410 nm) of the reduced coenzymes resemble either those of 'free' NAD(P)H in solution or of NAD(P)H 'bound' to dehydrogenases, such as alcohol dehydrogenase[2]. We have also reported[1] that those fluorescence properties are indistinguishable for the 'cytosolic' and 'mitochondrial' compartments when specific reduction of the coenzyme is brought about by use of appropriate inhibitors and substrates. The interpretation of these results, in terms of 'content' of NAD(P)H-binding sites[3] in the subcellular compartments of a given cell type, encounters difficulties due to the lack of information about activities of some dehydrogenases and properties of the binary and ternary complexes (including those resulting from combination of NAD(P)H to non catalytic proteins)[1]. However, among these un-

Abbreviation: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

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certainties, there is a growing body of evidence suggesting that, at least for some tissues like rat liver, NAD(P)H is mainly protein-bound[1,3,4].

Another approach to the problem of redox compartmentation in whole-cell preparations has now been studied and utilizes simultaneous measurements of fluorescence (F) and absorbance (A) of NAD(P)H, which allow calculations of F/A ratios to be made during kinetics of redox changes. A preliminary report of this work has been published[5].

MATERIAL AND METHODS

Ehrlich hyperdiploid ascites tumour cells of the Lettré strain (ELD) were grown in albino ICR mice by weekly transfer of 0.2 to 0.3 ml of ascites fluid. The cells were harvested between the 6th and the 8th day of growth, washed and resuspended in an isotonic saline medium (pH 7.4)[6]. The tumour cell preparations used for the experiments were always freed from erythrocytes.

Fluorescence and absorbance of NAD(P)H were measured simultaneously in the dual wavelength/split beam Aminco-Chance spectrophotometer with the Fluorescence attachment. The principle of the Fluorescence attachment is based upon the photomultiplier gain modulation circuit described elsewhere[7]. This circuit allows an equalization of the output of two photomultipliers, the first of which measures the light transmitted by the sample, the second, the fluorescence light emitted by the sample. The dual wavelength spectrophotometer is adjusted as usual for the transmission measurement so that the two light flashes from the reference and measuring wavelengths give equal outputs to the photomultiplier. A second photomultiplier at 90° to the first observes through an appropriate secondary filter. The two wavelengths for absorbance measurement excite fluorescence to different extents and 'leak' through the secondary filter to different extents. The wavelength at the maximum of fluorescence excitation (340 nm) gives the larger fluorescence signal, the reference wavelength (380 nm) excites very little fluorescence and is transmitted more by the secondary filter. The output of the response of the photomultiplier can be adjusted to be equal to these two signals by the above mentioned gain modulation circuit. Thereafter the electronics are conventional as described in the manufacturer's bulletin or previously[8]. The instrumentation is relatively simple and converts existing dual wavelength spectrophotometers to dual wavelength fluorometers in an economical and efficient manner. The same instrument was used for kinetics of flavoproteins. Difference absorption spectra were performed in a Perkin–Elmer Model 356 double beam/double wavelength absorption spectrophotometer. All the experiments were carried out at room temperature.

RESULTS AND DISCUSSION

Fig. 1 shows oxidation–reduction changes of nicotinamide nucleotides performed with Ehrlich–Lettré ascites tumour cells. Fluorescence (upper trace) and absorption (lower trace) of the coenzyme have been measured simultaneously, as described under Material and Methods. The excitation beam (at 340 nm) is also used as measuring light for dual wavelength absorption (with reference at 380 nm) and the emitted fluorescence is maximum near 435 nm. Cells have been supple-

mented with catalase (to allow O_2 evolution from added H_2O_2) and oxamate (to inhibit reoxidation of cytosolic NADH at the lactate dehydrogenase level)[9,10]. After the cells became anaerobic by endogenous substrates, a small amount of H_2O_2 (66 μM) is added and a normoxia–anoxia cycle is monitored (A). Owing to the lack of cytosolic reducing equivalents, in the absence of glycolytic substrates, this cycle is predominantly related to redox changes of 'mitochondrial' pyridine nucleotides. Addition of glucose to anaerobic cells results in the reduction of 'extramitochondrial' pyridine nucleotides (B), whose partial reoxidation by pyruvate is competitively inhibited by oxamate. The further addition of excess oxygen (3.3 mM H_2O_2) causes a biphasic response: a fast phase (C) is followed by a slow phase (D), the latter reaching steady state in about 60 sec.

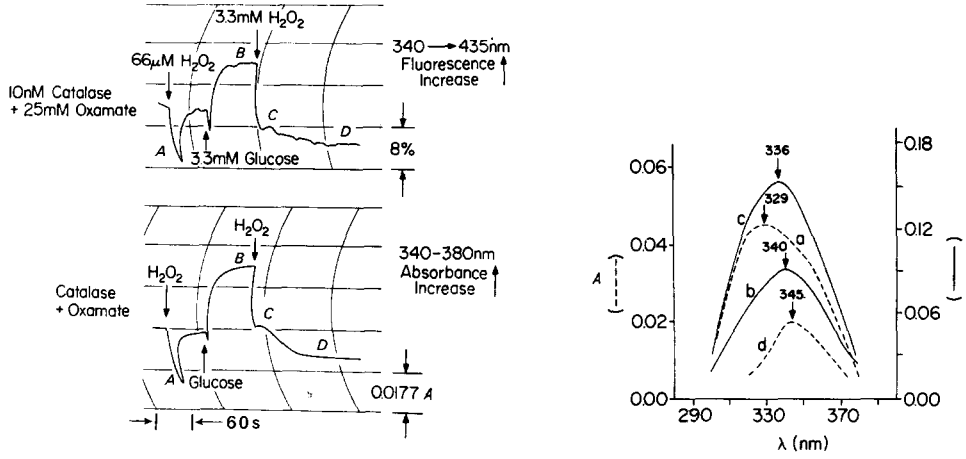

Fig. 1. Simultaneous recording of fluorescence and absorbance redox changes of nicotinamide nucleotides in Ehrlich–Lettré ascites tumour cells. The cells (5.5 mg dry wt./ml), pretreated with catalase and oxamate, were allowed to become anaerobic by endogenous substrates. Different amounts of H_2O_2 were then added for causing transient or permanent oxygenation of the cell suspension. Reduction of glycolytic NAD(P)$^+$ was obtained by glucose addition.

Fig. 2. Difference absorption spectra of nicotinamide nucleotides in intact cells under various metabolic conditions. a, anaerobic cells *minus* aerobic cells; b, anaerobic cells treated with glucose *minus* anaerobic cells; c, anaerobic cells treated with glucose *minus* aerobic cells; d was obtained by treating cells completely, as shown in Fig. 1, and adding TTFB only to one sample (measuring cuvette). Thus the spectrum of the last portion of the NAD(P)H absorbance trace (slow phase) is recorded (see also Fig. 3). The cell suspension concentration was 6.1 mg dry wt./ml.

Table I summarizes the F/A ratios for points A, B, C and D of Fig. 1. Distinction between mitochondrial and cytosolic NAD(P)H is afforded by the F/A ratios which are 2.65 $\pm$ 0.22 (6) and 1.46 $\pm$ 0.13 (6), respectively. Interestingly, such dichotomy is also observed with a single addition of excess H_2O_2 to the anaerobically glycolyzing ascites cells. Indeed the fast phase (C), shown in Fig. 1, has a F/A ratio of 2.24 $\pm$ 0.21 (6), whereas this ratio is lower (1.31 $\pm$ 0.12 (6)) for the slow phase (D). Even if binding of NAD(P)H to proteins usually also causes increase of the fluorescence quantum yield[11], the procedure of determining the F/A ratios may not allow, for the same reasons mentioned in the Introduction, a distinction between different degrees of coenzyme binding in the subcellular compartments. The use of such ratios, however, represents a 'probe' of redox compartmentation within the intact cell. Indeed, a

TABLE I

RELATIVE FLUORESCENCE EFFICIENCY OF NAD(P)H IN INTACT CELLS UPON DIFFERENT TREATMENTS

The F/A ratios have been calculated dividing the percentage fluorescence change by the percentage absorbance change. Suspensions of 5.5–6.0 mg dry wt./ml ascites cells have been used. The experimental conditions are those of Fig. 1. Values are given as means ± S.E.

Points in Fig. 1	*Conditions*	*Fluorescence/absorbance* ($n = 6$)
A	Limiting O_2	2.65 ± 0.22
B	Glucose	1.46 ± 0.13
C	Excess O_2	2.24 ± 0.21 (fast)
D		1.31 ± 0.12 (slow)

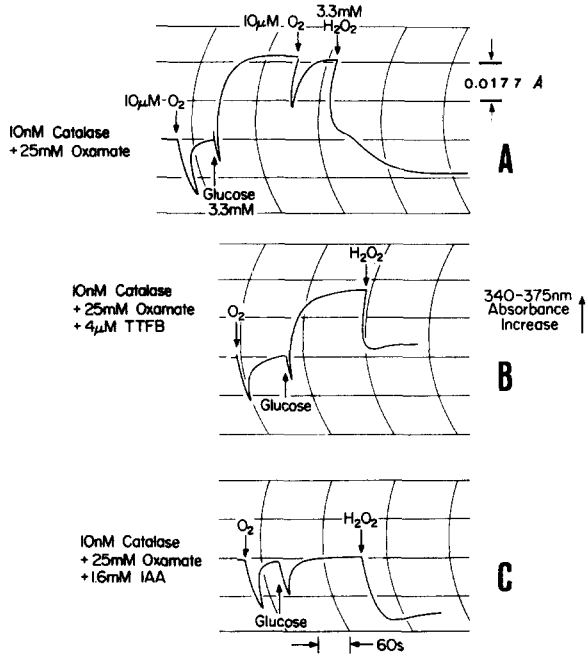

Fig. 3. Effect of TTFB and iodoacetate (IAA) on absorbance redox changes of nicotinamide nucleotides. The upper trace is similar to that shown in Fig. 1. TTFB abolishes the slow phase of nucleotides oxidation induced by excess of oxygen. The same is caused by iodoacetate which abolishes also the glucose effect. The cell suspension concentration was 5.2 mg dry wt./ml.

qualitative distinction may now be made between the fast and slow phases of nicotinamide nucleotide oxidation by their quantum fluorescence efficiency. In the presence of excess O_2 the nucleotides of the mitochondrial respiratory chain are first oxidized, followed, presumably, by intramitochondrial oxidation of cytosolic reducing equivalents. This conclusion may be drawn because the fluorescence properties (F/A ratios) for the two different compartments have been established.

Spectral evidence for difference in the absorption characteristics (peak position) of NAD(P)H in different intracellular compartments is presented in Fig. 2. The spectra are recorded as the difference between reduced and oxidized nicotinamide

nucleotides. a and b are the spectra of mitochondrial (anaerobic *vs* aerobic cells) and cytosolic (anaerobic cells treated with glucose *vs.* anaerobic cells) nicotinamide nucleotides, respectively. c (anaerobic cells with glucose *vs.* aerobic cells) is the sum of a and b, while d represents the spectrum of the slow-reacting NAD(P)H. As can be seen, mitochondrial reduced nicotinamide nucleotides (Trace a) have a maximum shifted towards lower wavelengths in respect to cytosolic nucleotides (Traces b and d). Spectrum d has been obtained by using the uncoupler 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) (see Fig. 3B). Fig. 3A serves as a control experiment for Figs. 3B and 3C. The slow phase reaches steady-state conditions after about 2 min (Fig. 3A) and is abolished by both TTFB and iodoacetate (Figs 3B and 3C).

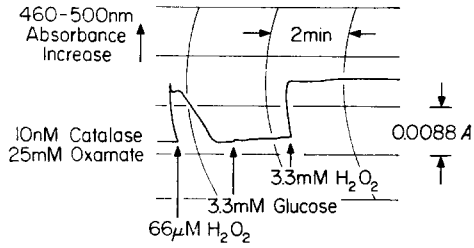

Fig. 4. The response of flavoproteins to addition of different amounts of oxygen and glucose. An upward deflection indicates oxidation. The cell suspension concentration was 8.1 mg dry wt./ml.

Finally, Fig. 4 shows redox changes of flavoproteins, measured at 460–500 nm. A normoxia–anoxia cycle is caused by addition of limiting O_2 to anaerobic cells. Glucose does not cause any change under anaerobic conditions, and the subsequent addition of excess of O_2 induces only a fast phase which, presumably, corresponds to the oxidation of mitochondrial flavoproteins. No slow phase is observed.

The data presented in Figs 3 and 4 strengthen the above-mentioned suggestion that in the fully oxygenated system the fast phase of nicotinamide nucleotides oxidation corresponds to a re-equilibration of electrons along the mitochondrial respiratory chain, whereas the subsequent slow phase is due to flow of cytosolic reducing equivalents into the mitochondria through an energy-dependent shuttle mechanism.

In conclusion, it is apparent that the binding of the NAD(P)H to the proteins of the mitochondrial space on the one hand, and to the proteins of the cytosol on the other hand, affords different fluorescent quantum yields. While it is not the purpose of this contribution to explore the nature of this difference in quantum yield, a procedure has been presented for the localization of the redox states in the mitochondrial and cytosolic compartments by measurements of the F/A ratios of intact cells. This probe of the redox compartmentation within the intact cell may be used for identifying the amounts of nicotinamide nucleotides involved in the separate redox changes of the two spaces, for the measurement of the kinetics of hydrogen transfer from cytosol to mitochondria and *vice versa* and finally, on a comparative biochemical basis, to explore differences in the compartmentation of NAD(P)H in different types of cells grown under appropriately different conditions.

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