SPECTROSCOPIC RESOLUTION OF THE "IRON-FLAVOPROTEIN TROUGH" OF THE RESPIRATORY CHAIN OF SUBMITOCHONDRIAL PARTICLES FROM *TORULOPSIS UTILIS*

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1. Introduction

We have previously shown [1, 2] that iron or sulphate limited growth of *T.utilis* leads to the loss of site I energy conservation and sensitivity to piericidin A. Both these properties may be restored by incubating the deficient cells aerobically with the nutrient that was previously growth limiting [1, 3].

Conditions were also described for the production of cells possessing site I energy conservation but lacking piericidin A sensitivity [2, 4]. Thus a dual involvement of non-haem iron protein in the segment of the respiratory chain between $NADH_2$ and the cytochromes was suggested. This conclusion was supported by EPR spectroscopy of phenotypically modified submitochondrial particles from *T. utilis* [2].

In this report, we describe studies on the "iron-flavoprotein" region of the visible spectrum of submitochondrial particles from *T. utilis*, and the use of iron or sulphate limited growth to modify the spectral characteristics.

2. Methods

Continuous culture conditions were as described previously [1, 2]. Iron limited cells were recovered aerobically with 1 μM iron with or without 100 μg cycloheximide/ml [1, 4]. Sulphate limited cells were recovered aerobically in low (< 50 μM) concentrations of sulphate [3]. Preparation of mitochondria and submitochondrial particles was as previously described [1, 2].

3. Results and discussion

The basic experimental design is illustrated in fig. 1. In trace (a), submitochondrial particles from glycerol limited cells were first treated with a low concentration of NADH. A cyclic decrease in absorbance at 470 nm was observed, which did not quite return to the original level. The pigment responsible for this small residual absorbancy change is unlikely to be a

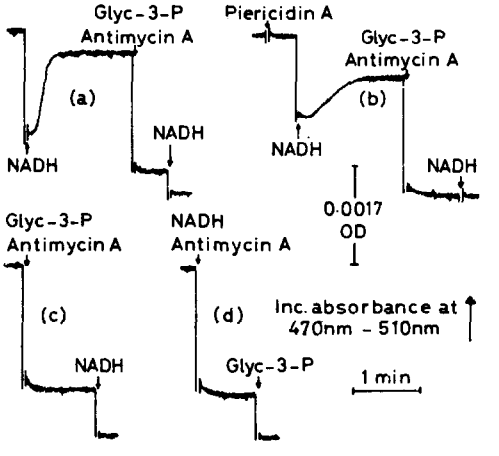

Fig. 1. Dual wavelength spectroscopy of submitochondrial particles from glycerol limited cells. Submitochondrial particles were suspended in 10 mM tris-SO_4 pH 7.4 to a final concentration of 1.0 mg/ml at 25° in each experiment. Reagents were added at the following final concentrations NADH 0.1 mM, glycerol-3-phosphate (glyc-3-P) 5 mM, antimycin A 1 μM, piericidin A 0.3 μM. Light path was 1 cm.

respiratory carrier and may be related to that found by Bois and Estabrook [5] and Minakami et al. [6]. Subsequent additions of glycerol-3-phosphate and antimycin A caused extensive bleaching at 470 nm and further addition of NADH reduced a pool of presumably low potential pigments, as they were not reduced by glycerol-3-phosphate. By the terms low and high potential, we mean mid point potentials similar to those of NADH and cytochrome *b* respectively [7]. Fig. 1 (b) shows that this latter pool is on the substrate side of the piericidin A sensitive site. Addition of NADH to particles approximately 70% inhibited by piericidin A resulted in a more sluggish cycle. The initial reduction was less extensive, and the ensuing reoxidation much less so. Addition of glycerol-3-phosphate and antimycin A caused a change in absorbance like that of fig. 1 (a), but no further decrease was found after further addition of NADH. Thus the low potential pool defined in fig. 1 (a) stays reduced following a cycle of NADH oxidation in piericidin A inhibited submitochondrial particles. A similar phenomenon has been observed in beef heart submitochondrial particles by Bois and Estabrook [5]. Prolonged incubation following a cycle of NADH oxidation in the presence of piericidin A will eventually lead to partial reoxidation of the low potential pool.

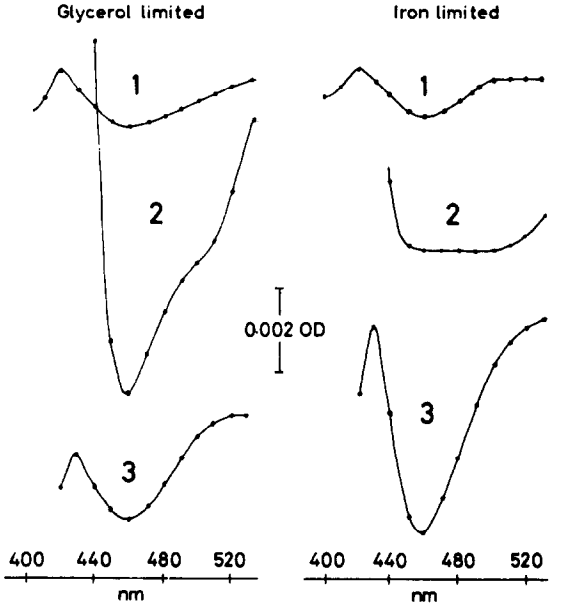

Fig. 2. Reduced minus oxidized difference spectra of species 1, 2 and 3 in submitochondrial particles from glycerol and iron limited cells. Spectra were plotted at 10 nm intervals with a dual wavelength spectrophotometer. Changes in absorbance are plotted with the higher wavelength of any chosen pair as the reference. In such plots no oxidized minus oxidized base line can be shown, but comparable experiments with a wavelength scanning spectrophotometer indicated that there is an isosbestic point at approx. 520 nm. Light path was 1 cm.

Submitochondrial particles from glycerol limited and iron limited cells were suspended at concentrations of 2.0 and 1.7 mg protein/ml respectively, in 10 mM tris-SO_4 pH 7.4 at 25°. Species 1, 2 and 3 difference spectra were obtained using the methods shown in fig. 1.

Table 1

Concentrations of species 1a, 1b, 2 and 3 in submitochondrial particles from glycerol and iron limited cells. For species 1a, 2 and 3, contents are expressed as 10^4 X absorbancy differences at 460–530 nm for a 1 mg protein/ml suspension of particles, using a 1 cm light path. For species 1b, the measuring wavelengths are 500–530 nm. The figures are averages of at least three separate determinations.

Species	Glycerol limited cells ($A \times 10^4$/mg protein/ml)	Iron limited cells ($A \times 10^4$/mg protein/ml)
1a	5.9	6.0
1b	3.6	< 0.4
2	28	< 1.0
3	12.5	30

The high redox potential pool, reduced by glycerol-3-phosphate in the presence of antimycin A may be divided into two: one reducible by NADH and one not. This is shown in figs. 1 (c) and (d). In (c), antimycin A inhibited particles were treated with glycerol-3-phosphate (reducing high potential components) and then NADH (reducing low potential components). Reversing the order of substrate addition (fig. 1 (d)) showed that NADH is capable of reducing some of the pool reduced by glycerol-3-phosphate, but not all.

In summary, therefore, these experiments indicate three species (1, 2 and 3) linked to the respiratory chain:

1) Low potential, reduced by NADH and located on the substrate side of the piericidin A sensitive site.

2) High potential, reduced by either NADH or glycerol-3-phosphate.

3) Specifically reduced by glycerol-3-phosphate.

Succinate is oxidized at extremely low rates by particles from *T. utilis* grown in continuous culture with glycerol as carbon source (less than 5 nmoles/min/mg protein in the presence of cytochrome *c*). Addition of succinate to antimycin A inhibited particles causes no significant reduction of cytochrome *b* or bleaching at 460 nm.

The experiments of fig. 1 were repeated at other wavelengths to obtain difference spectra of these species in submitochondrial particles from both iron and glycerol limited cells (fig. 2). The spectra of the low potential species 1 were characterized by a peak at around 420 nm and a trough with a minimum at 460 nm. The absorbancy decrease at 460 nm was comparable in both types of particle. However, in particles from glycerol limited cells, the trough extended to higher wavelengths than in those from iron limited cells. Thus, a considerable absorbancy decrease was obtained at 500–530 nm in the former, and none in the latter. Species 1 may, therefore, consist of at least two components, 1a, unaltered by iron limitation, maximally bleached at 460 nm, and 1b, removed by iron limitation, and maximally bleached at 500 nm. Changes in flavoprotein fluorescence accompanying reduction of species 1 are ascribed to lipoamide dehydrogenase, which is difficult to remove from these submitochondrial particles. Reduction of this flavoprotein will, therefore, contribute to the difference spectrum of species 1.

The high potential species 2, reducible by either NADH or glycerol-3-phosphate had a minimum at 460 nm and an inflection at about 510 nm, in particles derived from glycerol limited cells. In particles from iron limited cells, this high potential was undetectable. The difference spectrum of species 2 contains a contribution from cytochrome *b*. The height of the β band was estimated from the α band absorbancy change at 562–575 nm following addition of NADH or glycerol-3-phosphate and antimycin A. In particles from iron limited cells, the β band height fully accounted for the absorbancy change at 500–530 nm in the difference spectrum of species 2. The contribution of cytochrome *b* to the species 2 spectrum of particles from glycerol limited cells was only 10–20% of the total trough depth.

Ubiquinone reduction following addition of glycerol-3-phosphate and antimycin A was measured by the absorbancy decrease at 280–289 nm. From this, the absorbancy decrease at 465–510 nm due to ubiquinone was calculated. This amounted to at most, 4% of the species 2 absorbancy change in particles from glycerol limited cells.

Species 3, reduced only by glycerol-3-phosphate, had a difference spectrum similar in shape in both types of particle, and resembled that of a pure flavoprotein.

The concentrations of species 1a, 1b, 2 and 3 in the two preparations are shown in table 1. The figures for species 2 are corrected for the contribution from cytochrome *b*.

Submitochondrial particles from other phenotypic modifications of *T. utilis* were examined for the presence or absence of the species described above. Those from sulphate limited cells [2] resembled those from iron limited cells. Sulphate [3] and iron [1] recovery led to the reappearance of species 1b and 2. The latter, however, was present in much diminished concentration compared with the content of particles from glycerol limited cells. Mitochondria from iron limited cells recovered in the presence of iron and cycloheximide possess site I energy conservation but are insensitive to piericidin A [4]. Submitochondrial particles from these cells had no detectable species 2, but did recover species 1b.

The spectral properties and relationships with the growth conditions indicate that species 1a and 3 are flavoproteins, whereas 1b and 2 are non-haem iron proteins. Species 2 does not exhibit flavoprotein fluorescence, and its properties suggest that it can be identified with the non-haem iron protein reported by Hatefi and Stempel [8] rather than FpD_2 of Chance et al. [9] or ubiquinone [Albracht and Slater, 10].

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References

[1] P.A.Light, C.I.Ragan, R.A.Clegg and P.B.Garland, FEBS Letters 1 (1968) 4.
[2] R.A.Clegg, C.I.Ragan, B.A.Haddock, P.A.Light, P.B.Garland, J.C.Swann and R.C.Bray, FEBS Letters 5 (1969) 207.
[3] B.A.Haddock and P.B.Garland, in preparation.
[4] P.B.Garland, R.A.Clegg, P.A.Light and C.I.Ragan, in: Inhibitors: Tools in Cell Research, eds. Th.Bücher and H.Sies (Springer, Berlin, 1969) p. 217.
[5] R.Bois and R.W.Estabrook, Arch. Biochem. Biophys. 129 (1969) 362.
[6] S.Minakami, F.J.Schindler and R.W.Estabrook, J. Biol. Chem. 239 (1966) 2042.
[7] C.I.Ragan and P.B.Garland, European J. Biochem. 10 (1969) 399.
[8] Y.Hatefi and K.E.Stempel, Biochem. Biophys. Res. Commun. 26 (1967) 301.
[9] B.Chance, A.Azzi, I.Y.Lee, C.P.Lee and L.Mela. in: Mitochondrial Structure and Function, Vol. 17, eds. L.Ernster and Z.Drahota (Academic Press, London, New York, 1969) p. 233.
[10] S.P.J.Albracht and E.C.Slater, Biochim. Biophys. Acta 189 (1969) 308.