BBA 41104

The effect of light on flavoproteins in the presence of α-keto acids

WELLNER AND MEISTER[1] described the formation of a spectrally detectable species upon the exposure of fully reduced L-amino-acid oxidase (EC 1.4.3.2) to low oxygen concentrations in the presence of an excess of substrate. During our investigations on the mechanism of this reoxidation process, it was found that the rate of formation of this species, which was small in the dark, could be enhanced considerably by illumination at wavelengths below 500 nm. A larger amount of the species accumulated under anaerobic conditions, with low substrate concentration and in the presence of one of the products of the enzymatic reaction *e.g.* the α-keto acid.

The above-mentioned species is rather stable to further irradiation. Its spectrum shows a shoulder at 375 nm, maxima at 385 nm and 485 nm and absorption above 540 nm (Fig. 1A). It shows an EPR signal at $g = 2.00$*. The spectrum and EPR

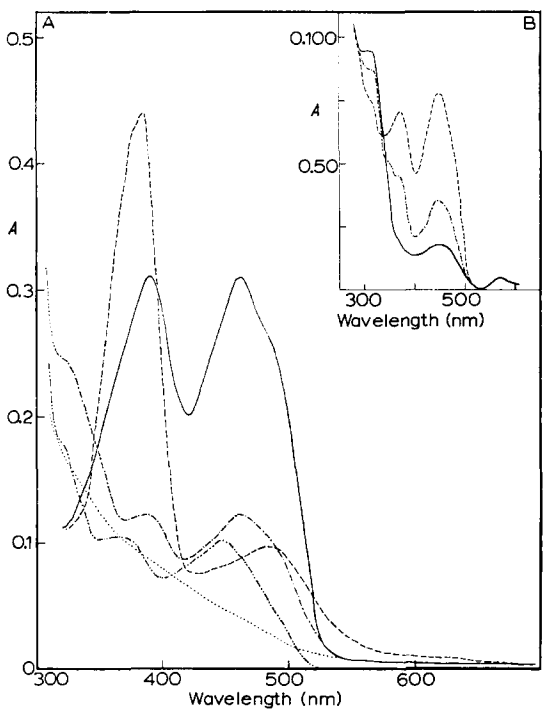

Fig. 1A. The effect of light on the spectrum of L-amino-acid oxidase in the presence of α-keto isocaproate. Temperature, 25°; anaerobic conditions; volume, 2.0 ml. ———, spectrum of $1.4 \cdot 10^{-5}$ M L-amino-acid oxidase and $5 \cdot 10^{-2}$ M α-keto isocaproate in 0.2 M Tris–HCl (pH 8.5); · · · · · ·, after the addition of 1.6 μmoles L-leucine; – – – –, after 2-min illumination with a 500-W xenon lamp; –·—·–, spectrum after 35-min illumination of the above-described mixture of oxidized enzyme and keto acid with a 500-W xenon lamp connected to a Zeiss monochromator (wavelength range 374–407 nm), followed by dialysis; –··—··–, same sample after deproteinization with perchloric acid and neutralization with KOH.

Fig. 1B. Effect of storage on the spectrum of a sample, purified by thin-layer chromatography after elution of the spot with R_F value of 0.56 with 1 M acetic acid. ———, after 1 day; –·—·–, after 1 week; – – – – – –, after 6 weeks.

* The EPR studies were done in collaboration with Dr. J. D. W. VAN VOORST.

signal of this species are similar to those of the intermediate, obtained during the anaerobic reduction of L-amino-acid oxidase with $Na_2S_2O_4$ (ref. 2) and possibly represent a semiquinoid form of the enzyme-bound FAD.

A similar species can be obtained when oxidized enzyme with excess of α-keto acid is irradiated under anaerobic conditions, but only when the reaction mixture is irradiated with light of about 320 nm (see Table I). Upon admission of oxygen the original spectrum is restored almost completely; only a small inactivation occurs.

TABLE I

THE EFFECT OF LIGHT OF DIFFERENT WAVELENGTHS ON THE REACTION OF L-AMINO-ACID OXIDASE WITH α-KETO ISOCAPROATE

The enzyme concentration was $3.1 \cdot 10^{-6}$ M, the α-keto isocaproate concentration was $1.2 \cdot 10^{-2}$ M. Solutions were made in 0.2 M Tris–HCl buffer (pH 8.5). Thunberg cuvettes were made anaerobic by repeated flushing with oxygen-free nitrogen. Irradiation was carried out with a stabilized 500-W xenon lamp connected to a Zeiss monochromator with slit set on 2 mm. The temperature was held constant at 25°. Spectra were recorded with the Cary 14 recording spectrophotometer. Intensities were determined with the ferrioxalate actinometer[9]. Activities were determined by the method of WELLNER AND MEISTER[10]. The values between parentheses are the % decrease in activity when no keto acid was present in the reaction mixture. No significant spectral changes were found in this case.

Wavelength range (nm)	*Quanta* $\times 10^{-18}$	*% Decrease in absorbance*				*% Decrease in activity*
		at 385 nm		*at 460 nm*		
		without oxygen	*after admission of oxygen*	*without oxygen*	*after admission of oxygen*	
237–243		0	0	0	0	0
275–285	2.0	6	19	56	25	48 (75)
293–307	2.6	−24	5	69	9	17 (66)
312–329	3.4	−35	4	70	6	9 (42)
330–351	3.2	−25	7	72	9	10 (14)
348–373	5.4	5	13	75	17	35 (27)
374–407	9.0	55	50	71	58	77 (29)
434–490	~15	7	6	7	6	11 (12)

A different reaction takes place upon irradiation with light of about 390 nm. The spectral changes resemble those observed upon partial substrate reduction (Fig. 1A). However, no reoxidation takes place upon admission of oxygen and an extensive inactivation is observed. The flavin remains in an apparent partially reduced state after dialysis and subsequent deproteinization.

After thin-layer chromatography on cellulose (solvent: *n*-butanol–acetic acid–water, 5:4:1, organic phase) four fluorescent spots were obtained. Following elution with 1 M acetic acid, three spots showed normal flavin spectra. The spectrum of the fastest-moving component (R_F value = 0.56) had a relatively low absorbance in the flavin region, increased absorbance above 540 nm and a maximum in the ultraviolet

at 260 nm (Fig. 1B). This spectrum gradually changed into a normal flavin spectrum upon storage at 4° in the dark, and the absorption above 540 nm disappeared. Rechromatography in the same solvent revealed a spot with a R_F value of 0.12, which was identical to that of pure FAD. Recombination with D-amino-acid oxidase apoprotein confirmed that the accumulated flavin was FAD.

Similar light-induced reactions occur with other flavoproteins. Upon irradiation of D-amino-acid oxidase (EC 1.4.3.3) with pyruvate at 320 nm, a species with an EPR signal at $g = 2.00$ is formed. The spectral properties, *i.e.* increased absorption at 380 nm, a shoulder at 400 nm, a maximum at 490 nm and increased absorption above 540 nm are similar to those of the species obtained with L-amino-acid oxidase under these conditions (*cf.* refs. 3, 4). Under the conditions used by us, no reaction occurs without pyruvate (*cf.* ref. 5). Upon irradiation at 390 nm the inactivation reaction as mentioned above, occurs. YAGI AND NATSUME[6] also described extensive inactivation following irradiation of L-amino-acid oxidase with light from a tungsten lamp.

In the case of succinate dehydrogenase (EC 1.3.99.1), oxaloacetate accelerates the light-induced formation of the EPR signal at $g = 2.00$ with respect to the signal at $g = 1.94$, 2.01.

An explanation of these effects must be found in the various reactions undergone by an α-keto acid when irradiated in its n-π^* transition band[7]. The triplet state has a long lifetime and is chemically very reactive. It can act as a strong hydrogen abstractor or else break up into reactive fragments such as carbanions. These carbanions may either reduce the unexcited flavin (at 320 nm) or react with the excited flavin (at 390 nm) in its triplet state to give a reduced addition compound. Preliminary experiments with D-amino-acid oxidase and [3-^{14}C]pyruvate show that in the thin-layer chromatogram the radioactivity is associated with the fast-moving flavin component, thus supporting the idea of an addition reaction. It is interesting to note that HEMMERICH, WALKER AND MASSEY[8] found that upon illumination of arylacetic acid and free flavin coenzymes, CO_2 was split off and reduced N-5 and C-4α addition compounds were formed. Upon admission of oxygen a rather stable semiquinone was formed, which slowly dealkylated and reoxidized to the original flavin. We have no evidence that under our conditions an addition compound is formed when the enzyme is replaced by free FAD, indicating that the formation of a complex between keto acid and enzyme is essential.

These investigations were carried out under auspices of The Netherlands Foundation for Chemical Research (S.O.N.) with partial financial aid from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.).

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Received February 16th, 1967

Biochim. Biophys. Acta, 131 (1967) 589–592

BBA 41106

Electron spin resonance of cytochrome b_2 and of cytochrome b_2 core

The ESR spectra of several haem proteins have been measured and information concerning their structures has been obtained[1–3]. Very recently, EHRENBERG AND BOIS POLTORATSKY[4,5] have made a detailed study, by this method, of cytochrome b_5 of liver microsomes at various pH. They have found, at neutrality, a main low-spin component with g values of 3.03, 2.23 and 1.43 and a small portion of a high-spin signal at $g = 6.2$. In alkaline medium (pH 12) they observed a narrowing of the low-spin absorption with limiting g values of 2.82, 2.28, 1.68 simultaneously with a considerable increase of the high-spin absorption.

It is known that cytochrome b_2 (L-lactate cytochrome c oxidoreductase, EC 1.1.2.3), first crystallized from yeast by APPLEBY AND MORTON[6], has a molecular weight of 183000 according to the method of ultracentrifugation diffusion[7] and contains two moles of haem and two moles of flavin mononucleotide. A low molecular weight derivative, the cytochrome b_2 core (noyau cytochromique b_2, *cf.* ref. 8) has been obtained either following trypsic hydrolysis ("t" type) or in the supernatant upon recrystallisation of cytochrome b_2 in the oxidised form ("s" type)[9]. These types, which appear to be very similar, were purified and it was confirmed that they have a molecular weight of about 11000 and carry one haem group[8,9] but no flavin. In many respects, this cytochrome b_2 core very closely resembles cytochrome b_5[10,5]: it has the same molecular weight, the same positions for the haem peaks in the absorption spectrum, the same absorbance coefficients and about the same redox potential.

The ESR study of cytochrome b_2 and of the cytochrome b_2 core was therefore performed to facilitate a comparison with cytochrome b_5 and also to elucidate whether the presence of flavin modifies the spectra of the former; by changing the pH, the dissociation of protons which control the state of the haem can be studied.

The cytochrome b_2 used in this experiment was crystallised by the method of APPLEBY AND MORTON[6]. Cytochrome b_2 core ("s" type) was prepared by the method of IWATSUBO *et al.*[9,11] and lyophilised. For the ESR measurements, crystalline cytochrome b_2 was packed in an ESR quartz tube (diameter, 3 mm) by centrifugation, and lyophilised cytochrome b_2 core was dissolved in 0.1 ml of 0.1 M phosphate buffer (pH 7.0). The pH was measured by a Beckman one-drop glass electrode after addition of acid or alkali. A Varian V-4502 EPR spectrometer was used at liquid-nitrogen temperature for the ESR measurements.

As shown in Figs. 1 and 2a, the ESR spectra of cytochrome b_2 and of cytochrome b_2 core at neutral pH are virtually identical and the g values of these two