FLUORESCENCE STUDY OF OXIDATION IN AZOALDOLASE

S. MONTI*, G. MONTAGNOLI, L. NANNICINI and R. FELICIOLI

C.N.R. – Laboratorio per lo Studio delle Proprietà Fisiche di Biomolecole e Cellule, Via F. Buonarroti 9, – 56100 Pisa, Italy

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1. Introduction

Diazonium salts have been extensively used in protein chemistry to introduce azochromophores covalently bound to the macromolecules, in the aim to study structure–function relationships both in enzymology [1] and in immunochemistry [2]. Diazonium salts are oxidizing agents [3]: side reactions can be expected in addition to coupling, especially when long reaction time or excess of reactant over the reducing residues of the protein are used.

In highly purified active azoaldolase [4] we have observed the presence of groups which fluoresce in the blue–violet region after excitation at 320–360 nm. The possibility of oxidative modification of aromatic sidechains in the protein is indicated by model experiments both of photoxidation of native aldolase and of oxidation of *N*-acetyl-tryptophanamide.

2. Materials and methods

Azoaldolases have been prepared by the standard procedure [4] already reported, by reacting rabbit muscle aldolase (EC 4.1.2.13) at neutral pH, when specifically adsorbed on phosphocellulose, with either diazotized *p*-aminobenzoate or diazotized 5-aminotetrazole. The modified protein was recovered by elution with the substrate, and purified either by Sephadex G-25 filtration or by dialysis against 0.1 N NaCl solution. The preparation and the handling of the modified enzyme has been done in the dark [5].

Oxidation of *N*-acetyl-L-tryptophanamide (Sigma) in dilute solution (18 μM) at neutral pH was carried out by means of freshly prepared diazotized *p*-aminobenzoate (1.3 μM) and followed by fluorescence spectroscopy. In the mean time, some formation of the azoderivative [6] was observed.

Irradiation of azoaldolase was performed in air in a box sheltered from diffuse light, while cooling the samples with air circulation. The light from a Quartz-Iodine 150 W lamp was collected and collimated to a parallel beam by pyrex condenser and filtered by a heat reflecting filter Calflex C Balzers and a broad band interference filter Filtraflex Kl Balzers (light centered at 400 nm, halfwidth 45 nm).

U.v. irradiation both of native aldolase and of *N*-acetyl-L-tryptophanamide was performed in air by cooling the sample with air circulation. The light from a high pressure HBO 200 W Hg lamp was collected and collimated to a parallel beam by quartz condensers and filtered by a solution of $CoSO_4$ and $NiSO_4$ prepared according to [7].

Absorption spectra have been recorded with the Unicam SP700 spectrophotometer and emission spectra with the Perkin-Elmer MPF3 spectrofluorimeter.

3. Results and discussion

When excited at 330 nm the azoaldolase prepared in the dark exhibits a fluorescence which is not proper of the native enzyme (fig.1, dotted curve). The emission cannot be ascribed to the azogroups; in fact fluorescence from acyclic azocompound has been found only in highly protonating solvents or in the case of structure which makes possible high concentration of

* Present address: Laboratorio di Fotochimica e Radiazioni di Alta Energia del C.N.R., Via de' Castagnoli 1, 40126 Bologna, Italy.

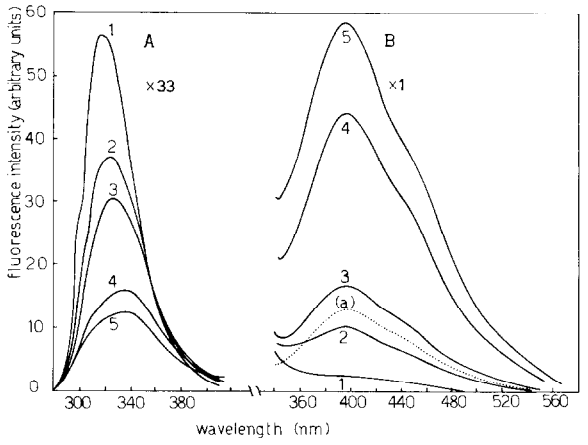


Fig.1. Emission spectra (uncorrected) of u.v. irradiated native aldolase (3.2 μM in 10 mM acetate buffer, pH 7.4. (A) Intrinsic fluorescence excited at 280 nm. (B) Fluorescence excited at 330 nm. (1) 0 min, (2) 5 min, (3) 10 min, (4) 30 min, (5) 50 min of irradiation. Dotted curve: emission spectrum of dark prepared azoaldolase (2.5 μM in 0.1 N NaCl, pH 6.9).

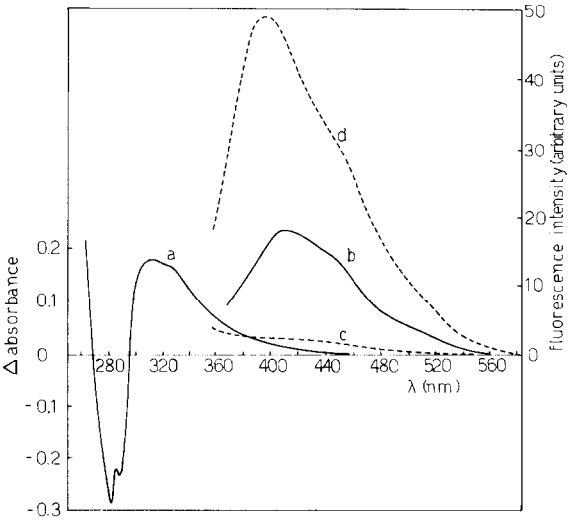


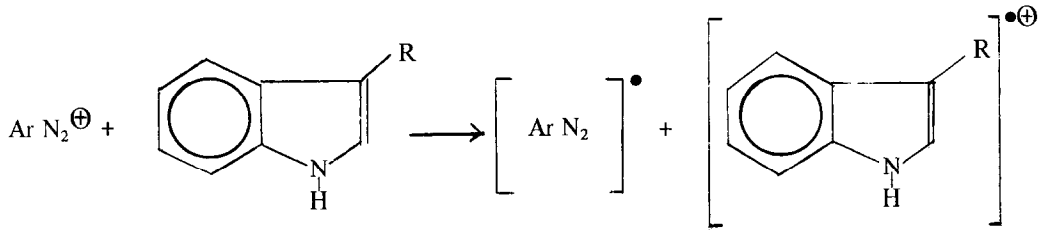
Fig.2. Stable fluorescent compounds from *N*-acetyl-L-tryptophanamide. Solid line: photolysis by u.v. light (15 min of irradiation); (a) difference absorption spectrum of the compound (0.109 mM in 4 mM phosphate buffer, pH 7.5). (b) emission spectrum of a solution 10.9 μM in the same buffer. Dotted line: emission spectra of the mixture of 18 μM *N*-acetyl-L-tryptophanamide and 1.3 mM diazotized *p*-aminobenzoate in 40 mM phosphate buffer, pH 7.1; (c) 15 min, (d) 120 min after mixing.

the tautomeric hydrazone forms [8]. Experimentally we have checked this statement with azoderivatives of tyrosine and histidine; also the free diazonium salt is not fluorescent in neutral aqueous solution.

In the recorded spectra the emission λ_{max} depends on the excitation wavelength, showing the presence of more than one component. The nature of these products can be clarified by observing that similar spectra have been obtained after photolysis of native aldolase in air equilibrated neutral solutions by irradiation with u.v. light (fig.1, full line curves). Various oxidation products of aromatic aminoacids are known to fluoresce in the observed wavelength range: kynurenine [9] and *N*-formylkynurenine [10] coming from the photolysis of tryptophan in the presence of O_2 [9], photoproducts of tyrosine [11,12] and of phenylalanine [12]. Nevertheless there are indications that in many cases degradation of proteins by light irradiation is due to photoxidative breakdown products of tryptophan [13]. Characteristic blue fluorescence has been observed in the insoluble brown proteins of the cataractous human lens, accompanied by a diminished tryptophane content and fluorescence [14]. Similar emission has been observed also by Dilley [15] and Pirie [16] in sunlight irradiated proteins, and correlated with tryptophan destruction.

In the present case tryptophan proved to be quite a good model to reproduce the phenomenon. Fig.2 reports the difference absorption spectrum of *N*-acetyl-L-tryptophanamide irradiated by u.v. light, showing the photolysis of the indole nucleus with appearance of products absorbing mainly in the 300–350 nm range. Some of these products are fluorescent; the emission spectrum of the irradiated sample is also reported. Similar fluorescence is obtained from *N*-acetyl-L-tryptophanamide simply after reaction with diazotized *p*-aminobenzoate in the dark (fig.2, dashed line).

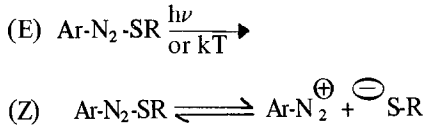
The similarity between the spectra of the stable fluorescent compounds obtained in the two cases supports the assumption of the presence of the same primary products of the aminoacid in the two different conditions. The initial step of the diazonium salt reduction has been formulated as a one-electron transfer from the reducing agent to the diazonium cation [3]. This mechanism would produce the same tryptophan radical cation as in the photoionization [17], the primary process in the photochemistry of aromatic aminoacids [18]:



By deprotonation the radical cation is known to give to the neutral radical [17], which could be intermediate to the stable fluorescent products. A free radical mechanism is known to operate also in the autoxidation of tryptophan [19,20]: the same intermediate has been hypothesized. It is possible that radical reactions after photoxidation of tryptophan play a role in the insolubilization and in the formation of non disulphide covalent crosslinks, observed in lens proteins after exposure to sunlight in vitro [21] and reproduced also with *S*-carboxy-methyl-lysozyme [15]. In the present case an enhancement of the scattered light in the fluorescence measurements of azoaldolase after irradiation was observed, presumably due to precipitation of the protein or to formation of aggregates.

Two points have to be discussed in relation to the azoaldolase. First, the fluorescence spectrum (fig.1a) has rather a low intensity. This is realized on the basis of internal quenching due to the diazothioether chromophores [22], formed on the protein by diazosubstitution on Cys-237 and Cys-287 [23]. The fluorescence intensity increases after irradiation with light only absorbed by the extrinsic chromophores; at the same time the absorbance due to the azogroups decreases, owing to photochemical changes occurring at the azo bond: E–Z isomerization at low doses of light (short irradiation time), and then photodegradation [22]. The variation in the fluorescence intensity immediately follows the modification in the absorbance, as it would be for the release of internal quenching (fig.3).

Secondly, in the degradation of the diazothioether chromophores on the protein, new diazonium ions are produced in situ:



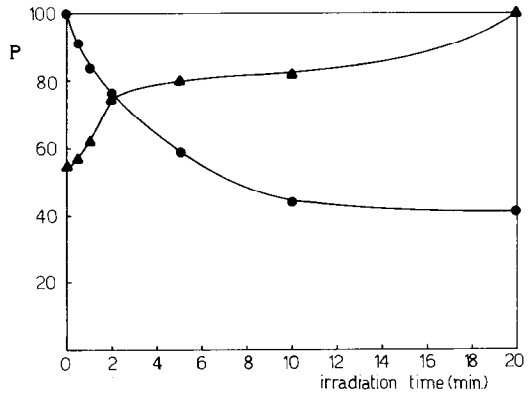


Fig.3. Spectral changes after light irradiation of azoaldolase (2.5 μM in 0.1 N NaCl, pH 6.9; light intensity 5.2 mW/cm²). P = (-●-) percent decrease in absorbance at 330 nm. (-▲-) increase of the fluorescence intensity at 400 nm (λ_{exc} = 330 nm), as percent of the value assumed after 20 min of illumination.

From the (E) isomer this production has a slow rate in the dark ($t_{1/2}$ = 39 h at 27°C), but it is very rapid under illumination [22]. These diazonium ions could in principle give rise to further oxidation reactions. In fact irradiated samples of azoaldolase showed a marked increase of intensity in the fluorescence excited at 330 nm, when kept in the dark for some hours; at the same time the enzyme is being inactivated.

All the discussed results give new insight in the azoprotein chemistry, by taking into account the oxidative properties of the diazonium cation. The involved reactions affect only few amino acid residues in the protein, so that it may be difficult to demonstrate the presence of modified groups by chemical analysis, although some data have been published [15]. The use of emission spectroscopy is a simple method of high sensitivity for monitoring stable fluorescent products which are present among the various products formed. It cannot be neglected that in azoaldolase

these reactions seem not to affect the kinetic properties of the enzyme [4,5], but in principle the chemical modifications produced could play a role in determining some other properties, like stability or immunological responses.

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