THE MODIFICATION OF TYROSYL RESIDUES IN L-ASPARAGINASES AND RIBONUCLEASE A WITH FREMY'S SALT. IMPLICATION OF TYROSYL RESIDUES IN THE ACTIVITY OF L-ASPARAGINASES FROM *E. COLI* B AND *ERWINIA CAROTOVORA*

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Received 26 August 1975

1. Introduction

Fremy's salt, potassium nitrosodisulphonate $[ON(SO_3K)_2]$ is a mild reagent for the conversion of phenols to quinones [1]. It has been shown to react in aqueous solution with tyrosine to yield dopachrome and with N-protected tyrosine derivatives to give the corresponding dopaquinones [2]. Thus the reaction of Fremy's salt with tyrosine and its derivatives yields the same initial products as tyrosinase [3] which has been extensively used for the modification of tyrosine residues in proteins [3–6]. The work reported here shows that Fremy's salt is also a useful reagent for the modification of tyrosyl residues in proteins to yield new chromophoric and fluorophoric groups characteristic of each protein although the nature of these, as in the case of tyrosinase treatment, has not yet been determined. Fremy's salt may prove to be a complimentary reagent to tyrosinase as two tyrosyl residues of ribonuclease are modified by Fremy's salt but not by tyrosinase [4,5], however, it may oxidise other amino acid residues, particularly tryptophan. The modification of 3–4 tyrosyl residues per subunit of the L-asparaginases from *E. coli* and *Erwinia carotovora* destroys enzymic activity, aspartic acid has been shown to exert a protective effect in the case of the latter enzyme suggesting that tyrosine may be involved in the active site of these enzymes.

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2. Experimental

E. coli B L-asparaginase (Merck) and *Erwinia carotovora* L-asparaginase (kindly provided by Dr H. E. Wade) were estimated at 280 nm using $E^{1\%}_{1\ cm} = 7.7$ [7] and 6.1 [8] respectively. Bovine pancreatic ribonuclease (4 × crystallised, BDH Limited) was determined at 280 nm using $E^{1\%}_{1\ cm} = 7.2$ [9]. Asparaginase activities were measured at pH 8.5 using a pH-stat (S. R. Allsopp, Ph. D. thesis, University of East Anglia, 1975). Fremy's salt was prepared by the literature method [1] taking care to keep the pH above 9, its concentration was estimated at 545 nm, $\epsilon = 20.8$ [10].

3. Results

The absorption spectra of all three enzymes changed progressively during treatment with 1×10^{-3} M Fremy's salt, pH 8.5. The final spectra after 24 h reaction are shown in fig.1, changes in extinction coefficient occurred at the 280 nm maximum and new bands appeared at longer wavelength, these are detailed in table 1. In addition to the new absorption bands the fluorescence spectra of the modified enzymes exhibited new bands in both emission and excitation spectra, table 1, although the original protein fluorescence at 303 nm in the case of the tyrosine emission of ribonuclease and *Erwinia carotovora* L-asparaginase and 318 nm from the tryptophan residues of *E. coli* L-asparaginase could still be detected.

Amino-acid analysis of the modified enzymes

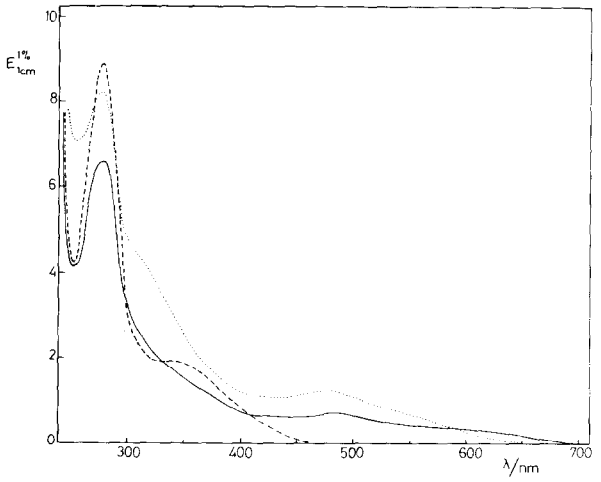

Fig.1. The spectra of Fremy's salt modified ribonuclease (· · · · ·), *Er. carotovora* L-asparaginase (– – –) and *E. coli* L-asparaginase (——). Reaction conditions: 1 × 10^{-3} M Fremy's salt, pH 8.5, μ = 0.1 carbonate buffer, 25°C for 24 hours.

revealed that major changes had occurred in the tyrosine content which was reduced by the following amounts, *E. coli* L-asparaginase 37%, ribonuclease 32%; *Er. carotovora* L-asparaginase 27%. Neither of the modified L-asparaginases had detectable activity on L-asparagine, the activity of modified ribonuclease has not been investigated.

The modification of *Er. carotovora* L-asparaginase was studied in more detail. The increase in absorbance of the 350 nm band and the loss of enzymic activity were measured as a function of time, both processes followed first order kinetics, fig.2a, yielding rate constants of 3.3 ± 0.3 × 10^{-5} sec^{-1} for the absorbance

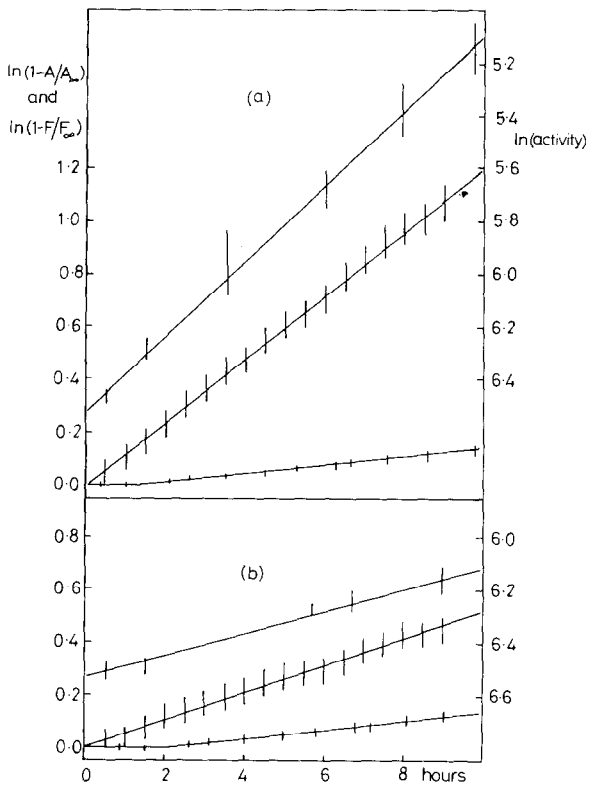

Fig.2. First order plots for the modification of *Er. carotovora* L-asparaginase by 1 × 10^{-3} M Fremy's salt in pH 8.5, μ = 0.1 carbonate buffer at 25°C. (a) Without aspartic acid. (b) 10^{-2} M aspartic acid. In both (a) and (b) top line is activity loss right-hand scale, centre line is absorbance increase at 350 nm and bottom line is fluorescence increase at 420 nm, both left-hand scale.

Table 1
Absorption and fluorescence maxima of Fremy's salt modified enzymes

Enzyme	Absorption maximum (nm)	Fluorescence maximum (nm) emission	excitation
L-asparaginase	280	303	278
(*Erwinia carotovora*)	350 (sh)	420	278 w, 345
L-asparaginase	280	318	280
(*E. coli*)	480, 600 (sh)	460	285 w, 365
Ribonuclease	280	303	278
	310 (sh), 475	472	293 w, 378

sh = shoulder, w = weak

change and $3.9 \pm 0.3 \times 10^{-5}$ sec^{-1} for the loss of activity. The presence of 10^{-2} M L-aspartic acid, an inhibitor of L-asparaginase [11], reduced both the rate of loss of activity ($k = 1.2 \pm 0.2 \times 10^{-5}$ sec^{-1}) and rate of increase in absorbance at 350 nm ($k = 1.4 \pm 0.2 \times 10^{-5}$ sec^{-1}) approximately three-fold as the decreased slopes of the first order plots in fig.2b demonstrate. The total increase in absorbance at 350 nm was unaffected by the presence of aspartic acid. The fluorophore emitting at 420 nm only became evident after a lag-period which was prolonged by the presence of aspartic acid (fig.2). The subsequent rate of production of the fluorophore was unaffected by aspartic acid and proceeded at a much slower rate than the increase in absorbance.

4. Discussion

The spectra of the Fremy's salt modified proteins all exhibit different features; the complexity of the spectra, which almost certainly reflect a variety of products in each case, is stressed by the non-coincidence of the absorption and excitation maxima. This suggests the presence of weak unresolved bands in the absorption spectra possibly due to minor products. There are some similarities with the spectra of tyrosinase modified proteins which have been reported to show an increase in absorption throughout the spectrum [3] accompanied by a new band at 350 nm [6], such a band is prominent in the spectrum of the Fremy's salt modified *Er. carotovora* L-asparaginase and is possibly present in the spectrum of modified ribonuclease. No band at 390 nm, which would have been characteristic of dopaquinone [6], was detected even in the early stages of the reaction of the enzymes with Fremy's salt although this is expected to be the initial product [2]. It is probable that the quinone reacts rapidly with neighbouring nucleophilic groups on the enzymes to yield products which have absorption spectra and, after further reaction, fluorescence spectra dependent on the nature of those groups. Thus the variety of spectra observed may reflect the disposition of nucleophilic sidechains around the reactive tyrosyl residues in the enzymes.

The spectral changes occurring with the *Er. carotovora* enzyme are probably the simplest although even here the production of the fluorophore was slower than the generation of the absorbance at 350 nm and was thus produced in a subsequent reaction. The kinetic study showed that the rates of production of the 350 nm band and the loss of activity were the same within experimental error and the association of these processes was emphasised by the equivalent reduction in their rate constants by aspartic acid. The protective effect of aspartic acid suggests competition with Fremy's salt at an active site tyrosyl residue although a more subtle protective mechanism may be operating [11]. Total loss of activity was observed in *E. coli* L-asparaginase and *Er. carotovora* L-asparaginase which had lost four of the eleven [7] and three of the twelve [12] tyrosyl residues per subunit respectively, the presence of at least one tyrosyl residue in the active site region of both enzymes is likely. Evidence from modification studies has previously suggested that tyrosine is at the active site of *E. coli* L-asparaginase [13].

The tryptophanyl residues of *E. coli* L-asparaginase, one per subunit [7], are not affected by Fremy's salt as judged by their unchanged fluorescence at 318 nm, although *N*-acetyltryptophan amide is oxidised under similar conditions (Arrieta, J. E. and Homer, R. B., unpublished). This confirms the inaccessibility of the tryptophanyl residues to solvent which had previously been suggested from their fluorescence spectra [14].

Ribonuclease is not modified by tyrosinase [4,5] whereas Fremy's salt modifies two of the six tyrosyl residues. In this case the small size of Fremy's salt and possibly its dinegative charge may give it access to tyrosyl residues which are sterically inaccessible to tyrosinase.

Fremy's salt is a free radical giving a three line ESR spectrum characteristic of nitroxides which can be used to follow its interaction with proteins, no new radical products were observed with the enzymes studied. Identification of the chromophoric and fluorophoric derivatives reported on here may yield information about the environment of tyrosyl residues in proteins.

Acknowledgements

We should like to thank Mr J. Slade (MRE, Porton Down) for running the amino-acid analyses. This work was supported by an extramural research contract from the Ministry of Defence. J.E.A. thanks the British Council for support.

References

[1] Zimmer, H., Lankin, D. C. and Horgan, S. W. (1971) Chem. Revs. 71, 229–246.
[2] Dukler, S., Wilchek, M. and Lavie, D. (1971) Tetrahedron 27, 607–614.
[3] Sizer, I. W. (1953) Adv. in Enzymology 14, 129–161.
[4] Cory, J. G. and Frieden, E. (1967) Biochemistry 6, 121–126.
[5] Lissitzky, S. and Rolland, M. (1962) Biochim. Biophys. Acta 56, 95–110.
[6] Yasunobu, K. T., Peterson, E. W. and Mason, H. S. (1959) J. Biol. Chem., 234, 3291–3295.
[7] Ho, P. P. K., Milikin, E. B., Bobbitt, J. L., Ginnan, E. L., Burck, P. J., Frank, B. H., Boeck, L. D. and Squires, R. W. (1970) J. Biol. Chem., 245, 3708–3715.
[8] Marlborough, D. I., Miller, D. S. and Cammack, K. A. (1975) Biochim. Biophys. Acta 386, 576–589.
[9] Bigelow, C. C. and Sonenberg, M. (1962) Biochemistry 1, 197–204.
[10] Murib, J. H. and Ritter, D. M. (1952) J. Am. Chem. Soc. 74, 3394–3398.
[11] Citri, N. and Zyk, N. (1972) Biochemistry 11, 2103–2109.
[12] Cammack, K. A., Marlborough, D. I. and Miller, D. S. (1972) Biochem. J. 126, 361–379.
[13] Menge, U. and Jaenicke, L. (1974) Hoppe-Seyler's Z. Physiol. Chem., 355, 603–611.
[14] Shifrin, S., Luborsky, S. W. and Grochowski, B. J. (1971) J. Biol. Chem. 246, 7708–7714.