Synthesis and Characterization of Covalent Flavin–Papain Complexes with Sulphur in the Sulphide and Sulphone Oxidation States

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Summary Two 2′,3′,4′,5′-tetra-acetylriboflavin derivatives of papain, differing in the oxidation state of the sulphur atom of Cys-25 which anchors the coenzyme species to the enzyme, have been synthesized by modification of the

active site of papain with 8α-bromo-2′,3′,4′,5′-tetra-acetylriboflavin under different conditions.

A MAJOR goal of our research is the conversion of simple enzymes which are hydrolytic catalysts into species which can catalyse a wide variety of synthetically important reactions including oxidation–reduction, transamination, and decarboxylation. We have attempted to prepare coenzyme analogues containing reactive functional groups, allowing them to be attached at or on the periphery of the active sites of readily available, easily purified, and stable enzymes. Recently, we described[1] the preparation of flavopapains (**1**) and (**2**) by the reaction of the SH group of the active site residue Cys-25 in papain with the brominated flavins (**3**) and (**4**). We report now that two discrete flavopapains differing in the oxidation state of the sulphur atom of the Cys-25 residue can be isolated following modification with the reagent (**4**) under different conditions. Furthermore, we have found that the structure of the flavopapain originally reported to be (**2**) is, in fact, (**5**), the sulphone form.

A solution containing papain (*ca.* $1{\cdot}5 \times 10^{-5}$ M, 10 ml), purified by affinity chromatography on a column of Gly–Gly–Tyr–Arg covalently bound to Sepharose,[2] was mixed with a 15-fold excess of 8α-bromo-2′,3′,4′,5′-tetra-acetylriboflavin[3] (**4**) dissolved in 0·5 ml of acetone and allowed to react for 2—3 h at room temperature. After addition of a 10-fold excess of cysteine and a further 0·5 h, the treatment of the enzyme with (**4**) was repeated, followed by dialysis against water at 4 °C for 3 days. The spectral properties of the resultant modified papain (**5**), in particular, the wavelength of the absorption maximum in the region 350—370 nm, and the relative intensity of the fluorescence emission, were different from those of the natural flavoenzymes which are known to contain a sulphide linkage between a Cys residue and the 8-CH_2 group of riboflavin, as shown in Table 1. It can be seen from Table 1 that these properties corresponded closely to those of the oxidized (sulphone) forms of naturally occurring enzymes such as hepatic monoamine oxidase[4,5] and chlorobium cytochrome C_{553}[6] in which the sulphide group linking a Cys residue to the coenzyme has been oxidized. To test the hypothesis that

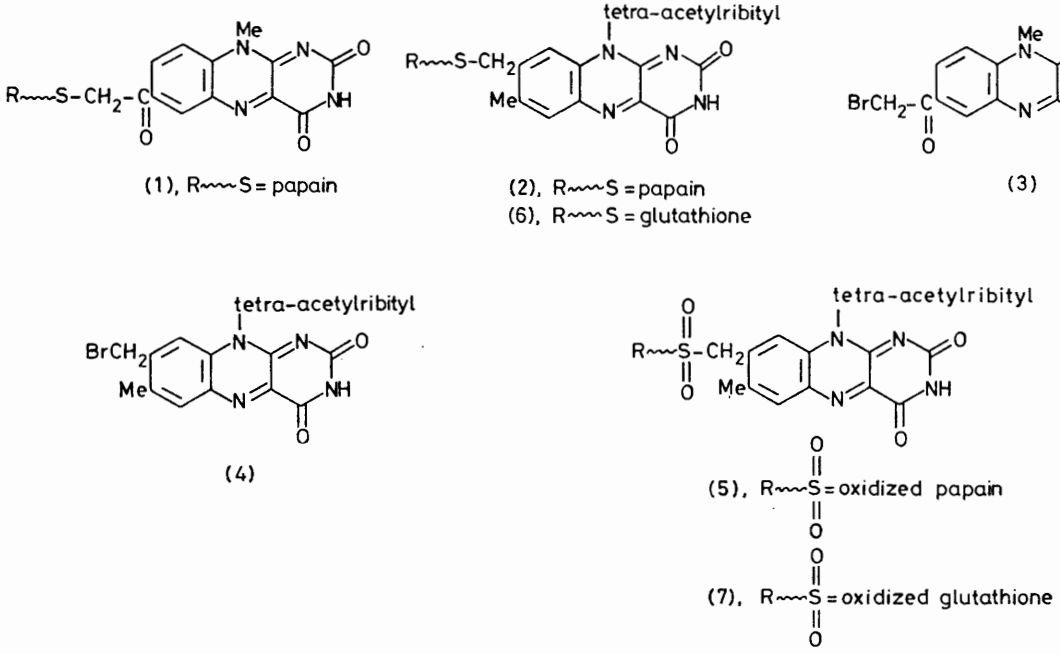

TABLE 1. Spectral properties of covalently bound flavins

Species	Oxidation state of S	λ_{max}/nm Neutral[d]	λ_{max}/nm Cationic[e]	% Fluorescence[g]
(**2**)	–S–	449,357	f	10[h]
(**5**)	$-SO_2-$	449,350	f	60[h]
(**6**)	–S–	448,364	390,268	8[h]
(**7**)	$-SO_2-$	448,353	405(sh),374,266	68[h]
Hepatic monoamine oxidase[a]	–S–	448,367	395	10[i]
	$-SO_2-$[c]	448,354	400(sh),375	70—80[i]
Chlorobium cytochrome C_{533}[b]	–S–	449,366		6[i]
	$-SO_2-$[c]	452,352		70[i]

[a] Identification was performed on a pentapeptide derivative of the flavin. [b] Identification was performed on a peptide derivative of the flavin obtained from peptic digestion. [c] The sulphone was obtained by oxidation of the sulphide form with performic acid. [d] Measured in aqueous solution or in a 0·05 M Tris buffer at pH 7·5. [e] Measured in 6 N HCl solution. [f] Too unstable to be measured under these conditions. [g] The relative % intensity is independent of pH over the range pH 4—8. [h] % intensity of emission of fluorescence at 520 nm (excitation at 450 nm) relative to 2′,3′,4′,5′-tetra-acetylriboflavin (100%). [i] % intensity relative to riboflavin (100%).

TABLE 2. Amino-acid analysis data for flavoenzymes[a]

Species	Oxidation state of S	No. of amino-acid residues: Cysteic acid	Carboxymethyl cysteine	Half-cystine	Total cysteine[b]
(2)	–S–	—	5·9	0·9	6·8
(5)	$-SO_2-$	1·2	5·5	—	6·7
Papain	–SH	—	6·8	—	6·8

[a] Denaturation, reduction, carboxymethylation, and acid hydrolysis were performed as described in the text. [b] The expected total cysteine content is 7 residues (A. N. Glazer and E. L. Smith, in 'The Enzymes,' ed. P. D. Boyer, 3rd edn., vol. 3, Academic Press, New York, 1971, p. 502).

the sulphur linkage of the flavopapain was in the sulphone state, denaturation by guanidine chloride, reduction by mercaptoethanol, modification by iodoacetic acid, and finally, hydrolysis by 6N HCl at 110 °C for 40 h were performed. One mole of cysteic acid per mole of flavopapain, resulting from scission at the sulphone function, was found and amino-acid analysis showed the presence of an appropriate amount of carboxymethylcysteine (Table 2) thus confirming that sulphur was in the sulphone form.

In contrast, the flavopapain with structure (**2**) was obtained when a solution of papain (*ca.* $1{\cdot}5 \times 10^{-5}$ M, 10 ml) was mixed under nitrogen with a 15-fold excess of the dihydro form of (**4**) dissolved in acetone (1·0 ml) prepared *in situ* by reduction with a very large excess of *N*-benzyl-1,4-dihydronicotinamide. The modification reaction was allowed to proceed for *ca.* 6 h, after which exhaustive dialysis of the reaction mixture against water at 4 °C was carried out. The spectral properties of the flavopapain resulting from this modification procedure were in good agreement with those reported for naturally occurring flavoenzymes containing a sulphide linkage between the enzyme and the flavin unit (Table 1). Furthermore, amino-acid analysis (Table 2) carried out with this flavopapain under the same conditions as had been used for the flavopapain (**5**) resulted in the detection of six residues of carboxymethyl cysteine and one half-cystine, as would be expected if the structure of the modified enzyme produced by reaction with dihydro-(**4**) were (**2**). Moreover, oxidation of the flavopapain (**2**) by performic acid led to changes in the u.v.-visible and fluorescence spectra which showed that a species with the properties of flavopapain (**5**) had been produced.

The flavin derivative (**6**) of glutathione[7] in the sulphide oxidation state, corresponding to the flavopapain (**2**), was obtained by modification with (**4**) at pH 8 in 0·1 M phosphate buffer and that in the sulphone oxidation state (**7**), was prepared by performic acid oxidation of (**6**). Purification of both modified glutathiones (**6**) and (**7**) was performed by chromatography on DEAE-Sephadex A-25. As illustrated in Table 1, the spectral properties of the covalent flavin–glutathione species in the sulphide and sulphone oxidation states corresponded with those of the related flavopapains. Polarographic measurement of the half-wave potentials for the flavin-glutathione derivatives gave values of $E_{1/2} = -175 \pm 5$ mV and $E_{1/2} = -160 \pm 5$ mV (*vs.* normal hydrogen electrode) for (**6**) and (**7**), respectively, at pH 8·3.

The mechanism by which flavopapain (**5**) is produced in the modification of papain by (**4**) remains to be elucidated.[8]

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