Tyrosine and tyrosinate fluorescence of pig intestinal Ca^{2+}-binding protein

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The single tyrosine residue in both pig and cow intestinal Ca^{2+}-binding proteins fluoresces at 303 nm although the crystal structure of the cow protein shows a hydrogen bond between the hydroxy group of the tyrosine and glutamate-38 [Szebenyi & Moffat (1986) J. Biol. Chem. **261**, 8761–8777]. The latter interaction suggests that tyrosinate fluorescence should dominate the emission spectra of these proteins. A fluorescence difference spectrum, produced by subtracting the spectrum of free tyrosine from the spectrum of the protein, gives a peak at 334 nm due to ionized tyrosine. That this component of the emission spectrum is not due to a tryptophan-containing contaminant is shown by its elimination when the protein is denatured by guanidine and when glutamate-38 is protonated. We conclude that, in solution, the tyrosine residue in this protein interacts occasionally with glutamate-38 but that a permanent hydrogen bond is not formed.

INTRODUCTION

One of the many effects of cholecalciferol is to stimulate the synthesis of a low-M_r CaBP in the small-intestinal absorptive cells of several mammalian species (DeLuca & Schnoes, 1983). The amino acid sequences of the pig (Hofmann et al., 1979) and cow (Fullmer & Wasserman, 1981) proteins show that they belong to the troponin C superfamily of CaBPs (Barker & Dayhoff, 1979), which also includes calmodulin (Cheung, 1982), parvalbumin (Wnuk et al., 1982) and S-100 CaBP (Morero & Weber, 1982; Mani et al., 1982). The three-dimensional structures of parvalbumin (Moews & Kretsinger, 1975), cow intestinal CaBP (Szebenyi et al., 1981; Szebenyi & Moffat, 1986), troponin C (Herzberg & James, 1985; Sundaralingam et al., 1985) and calmodulin (Babu et al., 1985) reveal proteins consisting of two or four Ca^{2+}-binding domains, each of which is made up of a Ca^{2+}-binding loop of 12 or 13 residues flanked on both ends by α-helices of ten or more amino acid residues each. These 'EF-hand' binding domains (Kretsinger, 1972) are also recognizable in the amino acid sequences of the other members of the superfamily whose tertiary structures are not known (Kretsinger, 1980).

Some of the high-affinity CaBPs play a central role in transmitting the Ca^{2+} 'second message' (Rasmussen & Waisman, 1983) and have been studied extensively. However, the intestinal CaBPs have no proven biological function. Both the cow and the pig intestinal CaBPs have been scrutinized by a variety of spectroscopic techniques, including absorption, intrinsic fluorescence (O'Neil et al., 1982), lanthanide-enhanced emission (O'Neil et al., 1984), c.d. (Dorrington et al., 1978), ^1H n.m.r. (Shelling et al., 1983, 1985; Shelling & Sykes, 1985) and ^{113}Cd and ^{43}Ca n.m.r. (Vogel et al., 1985). The highly homologous (92% sequence identity) pig and cow intestinal proteins are of interest spectroscopically, as they contain no tryptophan and have only a single tyrosine and five phenylalanine residues. The three-dimensional structure of the cow protein has been determined (Szebenyi et al., 1981; Szebenyi & Moffat, 1986), and it has been possible to confirm and extend certain spectroscopic predictions about the environment of the single tyrosine residue. However, one of the results of the spectroscopic studies (O'Neil et al., 1982) is not in complete agreement with the crystal structure, and in the present paper we examine the tyrosine fluorescence of the protein in greater detail in an attempt to reconcile the fluorescence and diffraction data.

The hydroxy group of tyrosine in the ground state ionizes with a pK_a of 9.8 (Fig. 1, reaction a). The deprotonated tyrosine has an absorption maximum at 295 nm (Donovan, 1973) and an emission maximum at 345 nm as well as a considerably diminished quantum yield (Cornog & Adams, 1963). In the excited state the pK_a of deprotonation is expected at about pH 4 (Fig. 1, reaction b) (Sandorfly, 1953), but in spite of this the tyrosine emission is constant between pH 2 and pH 9 because the rate of depopulation of the excited state by emission (Fig. 1, reaction c) is much faster than that by ionization (Fig. 1, reaction b) (Feitelson, 1964). However, ionization of excited-state tyrosine is catalysed by strong bases such as phosphate and acetate (Rayner et al., 1978). In proteins it is expected that the charged forms of glutamic acid and aspartic acid and the uncharged form of lysine residues would be suitable hydrogen-bonding partners (Weber & Rosenheck, 1964; Feitelson, 1964; Cowgill, 1976; Longworth, 1981). Water, and other weak bases such as the hydroxy groups of serine and threonine and the amide groups of the peptide chain, asparagine and glutamine, are not expected to promote excited-state protolysis (Longworth, 1981). Thus some hydrogen-bonded tyrosine residues (usually in the
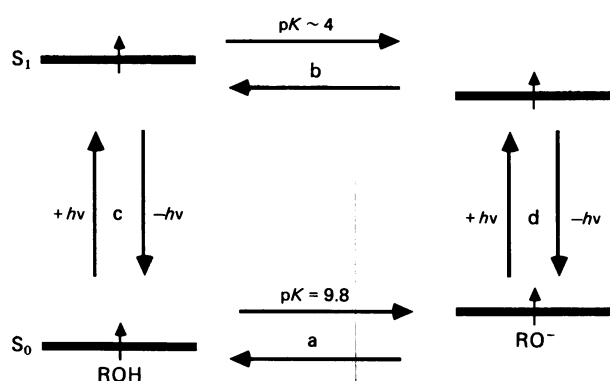
Fig. 1. Energy-level diagram for the two forms of tyrosine

The simplified energy-level diagram represents absorption and emission of quanta by tyrosine (ROH) and ionized tyrosine (RO⁻). Sandorfly (1953) has calculated that in the ground state (S_0) the phenolic oxygen atom carries a negative charge of 0.25. However, in the first excited state (S_1) this changes to a slightly positive value, although the charge on the hydrogen atom is nearly unaltered. The pK_a of the excited state, then, is considerably decreased, but, since the rate of emission (c) is greater than that of ionization (b), the emission is from the excited state of tyrosine even at neutral pH (Feitelson, 1964). However, in the presence of a proton-accepting base (b) emission from tyrosinate (d) can be observed at neutral pH.

interior of a protein) are expected to emit only at 345 nm (Longworth, 1981). Similarly, repetitive collisional interactions between the hydroxy group of tyrosine and a proton acceptor will diminish the quantum yield at 303 nm and increase it at 345 nm, depending upon the frequency and lifetime of transient hydrogen-bond formation. Several tyrosine-containing proteins have been shown to have emission spectra with peaks at wavelengths longer than 303 nm. Emission maxima or shoulders have been found in the following tryptophan-free proteins: parsley plastocyanin at 315 nm (Graziani *et al.*, 1974), Indian-cobra-venom cytotoxins at 345 nm (Szabo *et al.*, 1978), cow adrenodoxin at 331 nm (Lim & Kimura, 1980), human serum albumin at 345 nm (Longworth, 1981), ceratitus histone H1 at 325–350 nm (Jordano *et al.*, 1983) and ocomodulin at 345 nm (MacManus *et al.*, 1984).

The quantum yield of tyrosine fluorescence is also very sensitive to interactions of both the tyrosine carbonyl and the tyrosine phenyl groups with their environs. Important to this discussion is that location of non-hydrogen-bonded tyrosine in the hydrophobic interior of a protein (Giancotti *et al.*, 1980) or in an α-helix (Cowgill, 1968) enhances the quantum yield of tyrosine, whereas incorporation of tyrosine in a peptide bond diminishes the quantum yield (Cowgill, 1976).

X-ray-diffraction studies on cow intestinal CaBP (Szebenyi *et al.*, 1981; Szebenyi & Moffat, 1986) show that the single tyrosine residue is located in α-helix I, just before the beginning of the first Ca²⁺-binding loop. The hydroxy group of Tyr-16 is hydrogen-bonded to Glu-38 located in α-helix II, which follows the first binding loop. In agreement with this, the tyrosine fluorescence in the pig protein is greatly enhanced at acidic pH, presumably because the hydrogen bond is disrupted by protonation of Glu-38, whereas the α-helix remains intact (O'Neil

et al., 1982). On the other hand, the observation of tyrosine fluorescence at neutral pH (in both the pig and cow proteins; see Jones *et al.*, 1980) suggests that the interaction between Tyr-16 and Glu-38 is collisional rather than a permanent hydrogen bond.

MATERIALS AND METHODS

Pig intestinal CaBP was purified and the Ca²⁺-free form was prepared as described previously (Hitchman *et al.*, 1973; O'Neil *et al.*, 1982). Absorption spectra were measured with a Uvikon 820 recording spectrophotometer (Kontron Electronics, Redwood City, CA, U.S.A.) at 25 °C, and fluorescence excitation and emission spectra were taken with an Aminco SPF 500 spectrofluorimeter (American Instruments Co., Silver Spring, MD, U.S.A.) in the quantum corrected mode, also at 25 °C (O'Neil *et al.*, 1982). The determination of pH has been described elsewhere (O'Neil *et al.*, 1982).

Fluorescence difference spectra were produced as follows. Apo-CaBP (68.4 µM; A_{277} 0.115) and tyrosine (37.3 µM; A_{275} 0.053) were in 5 mm-square-cross-section cells, in 10 mM-Tris/HCl buffer, pH 8.0, containing 150 mM-NaCl. Solutions were excited at 278 nm with excitation and emission band widths of 4 nm. Spectra, consisting of 300–500 points, were recorded on cassette tapes at 40 nm/min and the recordings were transferred to computer storage discs. The emission spectrum from free tyrosine was normalized to the protein emission spectrum at different wavelengths, and putative tyrosinate spectra were calculated by subtracting the former from the latter.

RESULTS AND DISCUSSION

A tyrosine fluorescence emission spectrum is shown as spectrum a in Fig. 2(*a*) and has been discussed elsewhere (O'Neil *et al.*, 1982). In the present study we wished to investigate the degree of interaction, in solution, between Tyr-16 and Glu-38, by looking for evidence of tyrosinate emission. Spectrum b in Fig. 2(*a*) is an emission spectrum for free tyrosine at pH 8.0, and visual comparison of this spectrum with that of the protein shows that the latter is considerably broadened in the long-wavelength region. The possibility that the long-wavelength component arose from tyrosinate was investigated by subtracting the tyrosine emission from the mixture of tyrosine and putative tyrosinate emission (see the Materials and methods section). Fig. 3 shows that spectra with different shapes result depending upon the wavelength at which the emission from the protein and free tyrosine are normalized. Ideally, the tyrosine fluorescence should be normalized to the emission of the protein at a wavelength at which there is no emission from tyrosinate. The tyrosinate spectrum published by Cornog & Adams (1963) shows small but measurable emission even below 300 nm. Normalization below 285 nm will produce artifacts due to the Rayleigh light-scattering spectrum. These considerations have led us to conclude that the spectrum shown in Fig. 3(*a*) (normalized at 297 nm) is the best representative of the long-wavelength component of CaBP emission. This spectrum, which makes up about 15% of the total protein emission, has a maximum at 334 nm and is most probably due to tyrosinate (see below).
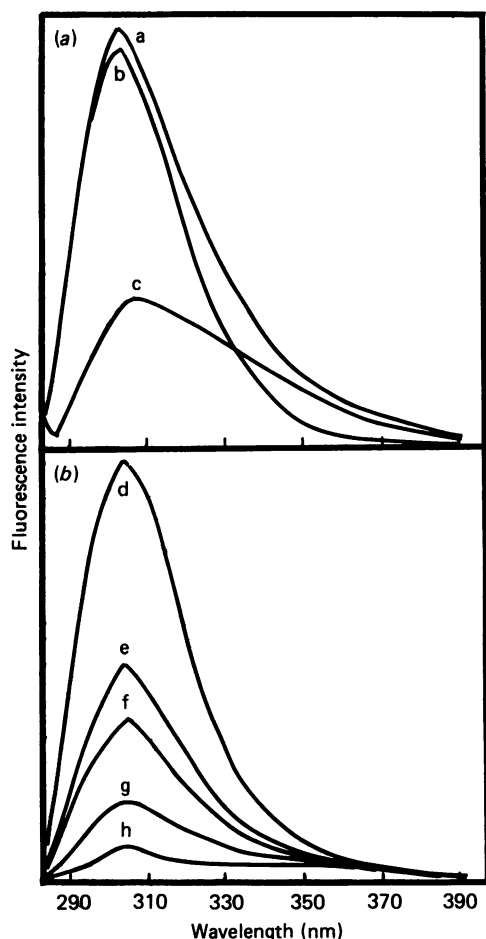
The absorption of the single tyrosine in CaBP is

Fig. 2. Emission spectra of apo-CaBP at various alkaline pH values in the presence and in the absence of 5 M-guanidine

(a) Apo-CaBP was pH-titrated as described previously (O'Neil et al., 1982). The protein was excited at 278 nm with a band-pass of 2 nm, and the emission was measured at 303 nm with a 5 nm band-pass. The quartz cells were 10 mm square in cross-section. The protein concentration was 60.7 μM (A_{277} 0.102) in 10 mM-Tris/HCl buffer containing 150 mM-KCl. The spectrum of tyrosine (78 μM; A_{277} 0.106) was normalized to the spectrum of CaBP by decreasing the sensitivity of the chart-recorder. Because blank spectra were not subtracted, Raman emission is clearly visible at 305–307 nm, when the tyrosine fluorescence is quenched. Spectrum a, CaBP, pH 7.8; spectrum b, tyrosine, pH 8.0; spectrum c, CaBP, pH 12.1. (b) CaBP (51.2 μM; A_{277} 0.086) in 10 mM-Tris/HCl buffer, pH 8.0, containing 5 M-guanidinium chloride and 150 mM-KCl was heated at 108 °C for 3 h. All other conditions were as described for panel (a). Spectra of the denatured protein were collected at 25 °C. Spectrum d, pH 7.87; spectrum e, pH 9.88; spectrum f, pH 10.08; spectrum g, pH 10.50; spectrum h, pH 11.01 (A_{277} 0.122). According to Edelhoch (1967), the ϵ_{295} for ionized tyrosine in 8 M-guanidine is 2480 M^{-1}·cm^{-1} (compared with 2330 M^{-1}·cm^{-1} in water), and this value was used to estimate that a minimum of 78% tyrosine ionization had occurred at pH 11. The fluorescence titration in guanidine was characterized by a pK_a of 9.8 and a Hill coefficient of unity.

red-shifted by about 2 nm compared with that of free tyrosine (see Fig. 1 in Dorrington et al., 1974). This is not unusual and is probably mainly due to hydrophobic

contacts of the phenyl ring (Szebenyi et al., 1981). A hydrogen bond between the Tyr-16 and Glu-38 would contribute only a small part of this shift, and so absorption spectroscopy cannot distinguish between a permanent and a transient interaction between the Glu-38 and Tyr-16 (Donovan, 1969). The absorption spectrum does, however, make it clear that in the ground state the tyrosine residue exists entirely in the protonated form. Furthermore, the absence of an absorption maximum at 288.5 nm and the absence of significant absorption at 295 nm shows that possible contamination by tryptophan is negligible. Nevertheless absorption spectroscopy is not sensitive enough to rule out the possibility that the emission at 334 nm is due to a tryptophan contaminant.

Excitation spectra of the Ca^{2+} form of CaBP, at pH 8.0, monitored at 303 and 317 nm (see Fig. 2; O'Neil et al., 1982) and at 340 nm (not shown) are all identical in shape, though the spectrum at 340 nm is red-shifted by about 3 nm and considerably diminished in intensity. These spectra show that the emission at all wavelengths arises from tyrosine, since a tryptophan contaminant would be expected to have a significant component in the excitation spectrum above 290 nm, where tyrosine does not absorb. Similarly, an emission spectrum excited at 290 nm (not shown), though considerably diminished in intensity, was identical in shape with those obtained by excitation at 278 nm, also suggesting that the long-wavelength component of protein fluorescence arises from tyrosinate and not tryptophan.

The hydroxy group of the tyrosine residue in CaBP ionizes with a pK_a of approx. 12 (O'Neil et al., 1982), highly unusual for an exposed chromophore. The explanation for this seems to be the hydrophobic contacts of the phenyl ring and the interaction between Glu-38 and the hydroxy group of the tyrosine (Szebenyi et al., 1981; Szebenyi & Moffat, 1986). However, the tyrosine fluorescence is quenched with a pK_a of 10.0, the best explanation for this quenching being transient proton transfer to a neutral lysine amino group (Lys-15 and/or Lys-19) (O'Neil et al., 1982). In accord with this interpretation, as the pH is elevated the relative proportion of the emission at 303 nm is diminished, whereas the fraction of the long-wavelength component of the emission spectrum increases although the total tyrosinate emission is quenched at very alkaline pH values (Fig. 2a, spectrum c). The pH-dependence of tyrosinate emission has not been directly examined, and it is possible to speculate that the latter is quenched at alkaline pH values (see Rayner et al., 1978). Indeed, this would help to explain the very low quantum yield observed for tyrosinate at pH 13 (Cornog & Adams, 1963). It would also help to explain the observation that in cow adrenodoxin, at alkaline pH, the putative tyrosinate emission maximum shifts from 331 to 345 nm and the quantum yield is greatly diminished (Lim & Kimura, 1980). Again, excitation spectra measured at 303 nm (not shown), at neutral pH, were identical in shape with those measured at 325 and 340 nm at alkaline pH, supporting the suggestion that all emission arises from tyrosine.

Comparison of spectra a in Fig 2(a) and d in Fig. 2(b) shows that the long-wavelength component of tyrosine emission is eliminated when the protein is unfolded by heat in 5 M-guanidinium chloride. The absence of tyrosinate emission at neutral pH and the determination, by absorption spectroscopy, of 78% ionization of the
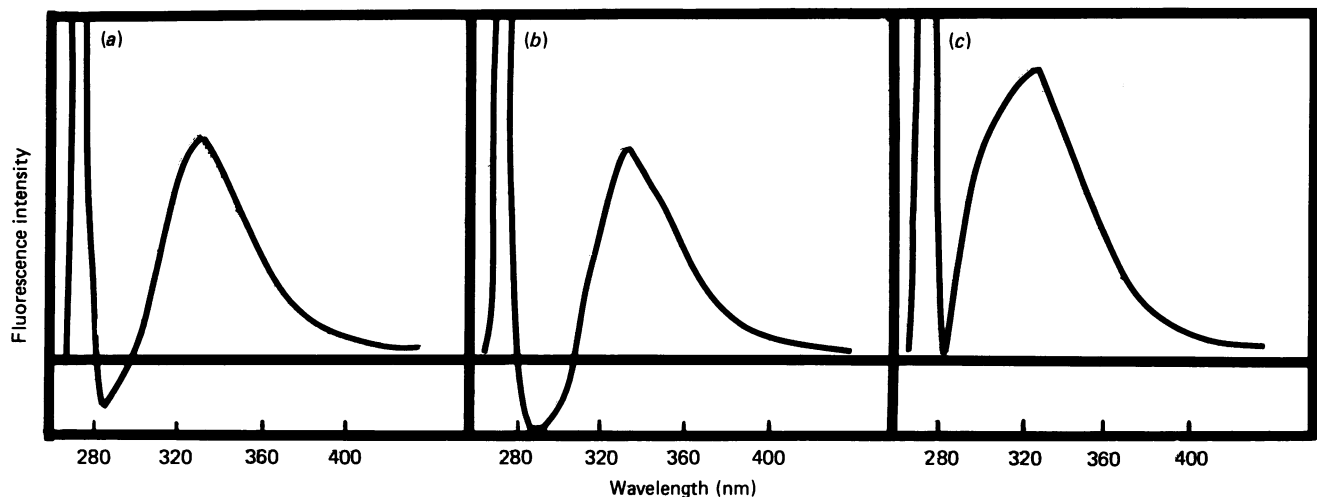
Fig. 3. Fluorescence difference spectra of CaBP and tyrosine

An emission spectrum, obtained by excitation of apo-CaBP at 278 nm, was normalized to a spectrum obtained by excitation of tyrosine at 278 nm. The difference between these spectra was calculated and plotted by computer. Normalization was at (a) 298 nm, (b) 310 nm and (c) 287 nm.

tyrosine at pH 11 shows that, in the unfolded protein, the interaction between Glu-38 and Tyr-16 and the hydrophobic contacts of the phenyl ring have been eliminated. This result conclusively rules out the possibility that the CaBP emission spectrum is contaminated by a contribution from tryptophan. Spectra e–h in Fig. 2(b) give no indication of any increase in tyrosinate emission due to proton transfer to a neutral lysine amino group at elevated pH values, despite the possibility that Tyr-16 might interact occasionally with Lys-15 even in the unfolded protein. That the Hill slope of the tyrosine fluorescence quenching is unity and the pK_a 9.8 (see the legend to Fig. 2) also indicates that only tyrosine ionization is responsible for quenching the tyrosine fluorescence.

We have shown previously (O'Neil et al., 1982) that the quantum yield of tyrosine is greatly enhanced at acid pH with a pK_a of 4.1, although the secondary structure of the protein is unchanged by acidic pH according to c.d. spectroscopy. Protein emission spectra at pH 3 (not shown) are devoid of tyrosinate emission. This suggests that Glu-38 is the proton acceptor required for the existence of tyrosinate emission (Fig. 1, reaction b) in CaBP.

CONCLUSIONS

The observation of tyrosinate emission in pig intestinal CaBP is additional evidence that an interaction between the hydroxy group of the Tyr-16 and Glu-38 does occur in solution. However, only 15% of the protein emission is tyrosinate, suggesting that, unlike in crystalline CaBP (Szebenyi et al., 1981; Szebenyi & Moffat, 1986), a permanent hydrogen bond does not exist in solution.

An interesting experiment would be the measurement of tyrosine emission from the crystal form of the protein. This might help clarify the uncertainties about the exact environmental conditions that are required for the observation of tyrosinate fluorescence. The reason that the emission maximum is at 334 nm, as opposed to the

345 nm observed for tyrosinate at pH 13 (Cornog & Adams, 1963), is not known. It may be that the wavelength at which the tyrosinate emission maximum occurs is dependent upon environmental factors. On the other hand, the maximum at 334 nm may be due to the technical difficulties of producing a small difference spectrum from two large-basis spectra.

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