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Fluorescence and circular-dichroism properties of pig intestinal calcium-binding protein (M_r = 9000), a protein with a single tyrosine residue

Joe D. J. O'NEIL, Keith J. DORRINGTON, David I. C. KELLS and Theo HOFMANN
Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8

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Spectral properties of pig intestinal Ca^{2+}-binding protein (CaBP) and its apoprotein have been examined by fluorescence, absorption and c.d. Direct fluorescence from some of the five phenylalanine residues is observed and excitation spectra show that there is also energy transfer from some phenylalanine residues to the tyrosine. Absorption and c.d. spectra show that the tyrosine hydroxy group does not ionize significantly below pH 12. Tyrosine fluorescence is reversibly quenched by a lysine residue with a pK of 10.05 in the Ca^{2+} form. At low pH the tyrosine fluorescence is enhanced with transitions with pK values of approx. 4.2. The c.d. spectrum of the Ca^{2+} form shows a decrease of the ellipticity band at 276nm with a transition similar to that of the fluorescence titration. The apoprotein, however, shows an additional transition with a pK of about 6. The results are interpreted in terms of the recently published structure of the cow intestinal CaBP [Szebenyi, Obendorf & Moffat (1981) *Nature* (*London*) **294**, 327–332]. The single tyrosine has a very high pK, although it apparently lies on the surface of the protein molecule.

The presence of a high-affinity CaBP in the small intestine of a variety of mammalian species has been reported by several investigators (Kallfelz *et al.*, 1967; Drescher & DeLuca, 1971; Hitchman & Harrison, 1972). This protein (M_r 9000) is distinct from the chicken vitamin D-dependent intestinal CaBP of M_r 27000 (Wasserman *et al.*, 1968). Molecular properties of the pig protein have been described previously (Dorrington *et al.*, 1974) and the spectroscopic effects of bivalent cation binding have been reported (Dorrington *et al.*, 1978). The amino acid sequences of the pig protein (Hofmann *et al.*, 1979) and of the cow protein (Fullmer & Wasserman, 1981) show 87% identity. The mammalian intestinal CaBP's are of interest because a comparison of their sequences with those of other high-affinity Ca^{2+}-binding proteins suggests that they are related through a common evolutionary ancestor and belong to the troponin C superfamily (Barker *et al.*, 1977; Barker & Dayhoff, 1979). This superfamily also includes the family of the parvalbumins, troponin C and the family of the ubiquitous calmodulin (Barker *et al.*, 1979). Kretsinger (1979) suggested that all of these Ca^{2+}-modulated proteins function as targets of the 'second messenger' Ca^{2+} (Rasmussen & Goodman, 1977), a role that has been amply demonstrated for calmodulin in a bewildering variety of reactions (for reviews, see Klee *et al.*, 1980; Cheung, 1980; Means & Dedman, 1980), and for troponin C in muscle contraction (Ebashi *et al.*, 1978; Perry, 1979; Weeds & Wagner, 1981). However, so far no physiological function has been demonstrated for the parvalbumins and for the mammalian intestinal CaBP's.

The pig and cow intestinal CaBP's are also interesting because they contain only a single tyrosine residue and are devoid of tryptophan. Previous studies (Dorrington *et al.*, 1978) showed that the 276nm c.d. band, originating from tyrosine, was highly sensitive to the presence of Ca^{2+}. This suggested that extended spectral studies might provide further insight into the relation between the tyrosine. residue and the Ca^{2+}-binding site. A preliminary report on the fluorescence properties of the cow protein has appeared (Jones *et al.*, 1980).

The present paper describes fluorescence and further optical absorption properties of the pig intestinal CaBP as a function of pH and Ca^{2+} binding.

Abbreviation used: CaBP, Ca^{2+}-binding protein.

Experimental

Materials

Pig intestinal CaBP was prepared by the method of Hitchman *et al.* (1973), except that a small amount of ^{125}I-labelled CaBP (Murray *et al.*, 1974)

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was added to the tissue homogenate to facilitate detection during chromatography. The purity of the protein was checked by amino acid analysis; this is a good criterion, because the protein lacks histidine, half-cystine and methionine. The absorption and fluorescence spectra were also used to check for the absence of tryptophan.

Ca^{2+} was removed from the protein on a single Sephadex G-25 column (2cm × 35cm) (Pharmacia, Montreal, Quebec, Canada) poured over Chelex-100 (2cm × 2cm) (Bio-Rad, Richmond, CA, U.S.A.) and equilibrated in 10.0mM-Tris/HCl buffer, pH8.0, and NaCl or KCl as specified in the text. A solution of the protein containing 10.0mM-EGTA, pH8.5, was passed over the column and the protein fractions were collected. These fractions were shown to contain less than 1mol of Ca^{2+}/30mol of protein by atomic-absorption spectroscopy (Perkin–Elmer 303, Norwalk CT, U.S.A.). Since the apoprotein exhibits a characteristic c.d. spectrum in the near-u.v. range (Dorrington *et al.*, 1978), this property was used routinely as the criterion for adequate removal of Ca^{2+}. The Ca^{2+} form was obtained by adding small portions of 50mM-$CaCl_2$ to a solution of the protein up to a Ca^{2+}/protein ratio of 2.0. The extent of Ca^{2+} saturation of CaBP was also checked by its characteristic c.d. spectrum in the near-u.v. range.

Methods

Protein concentration. This was determined spectrophotometrically at 277nm by using a molar absorption coefficient of $1681\,M^{-1}\cdot cm^{-1}$ ($A^{0.1\%}_{1cm,277}$ 0.191). This constant was calculated from the protein concentration determined with a Hilger–Watts Rayleigh Interference Refractometer (see Doty & Geiduschek, 1953).

pH titrations. A Radiometer type TTT1b (Radiometer, Copenhagen, Denmark) pH meter was standardized to ±0.03 unit in the pH range of interest with three buffer solutions of known pH (Fisher Scientific Co., Fairlawn, NJ, U.S.A.). The pH titrations below pH8 were done by adding small volumes of dilute HCl or NaOH to 2.0ml of protein solution in a cuvette. Because of the Na^+ effect on the pH electrodes (Donovan, 1973), dilute KOH solutions were used for titrations above pH8. The total volume changes due to addition of titrants were seldom greater than 5%, but when this value was exceeded, spectral values were corrected for the dilutions.

Spectroscopy. U.v.-absorption spectra were determined in a Beckman DU-8 single-beam spectrophotometer equipped with a temperature-controlled cell holder that was set at 25°C (Beckman Instruments, Palo Alto, CA, U.S.A.).

C.d. spectra were measured at room temperature on a J-41A spectropolarimeter (Japan Spectroscopic Co.) equipped with a built-in data processor. Spectra in the near-u.v. absorption region (320–250nm) were signal-averaged four times to reduce noise. Results are expressed as molar ellipticity (Adler *et al.*, 1973) using a mol.wt. of 8799 (Hofman *et al.*, 1979).

Fluorescence excitation and emission spectra were obtained by using an Aminco SPF500 spectrofluorimeter (American Instrument Co., Silver Spring, MD, U.S.A.) in the quantum-corrected mode. The jacketed cell compartment was maintained at 25°C by a circulating constant-temperature water bath. Other pertinent information is given in the legends to the Figures. Fluorescence yields for tyrosine were calculated by using the equation from Cowgill (1968). Solutions of L-tyrosine were prepared daily in 10mM-Tris/HCl buffer, pH8.0, containing 150mM-NaCl or -KCl.

Rayleigh scattering was eliminated from fluorescence spectra by using a computer calculation. A numerical method was developed that was based on the observation that scattering peaks obtained with glycogen were symmetrical around the excitation wavelength. Thus the low-wavelength half of the curve, which did not overlap with the fluorescence emission curve, could be subtracted from the overlapping half to give the corrected curve.

Amino acid numbering

The amino acid residue numbering of both pig and cow intestinal CaBPs used in the present paper is based on the sequence of the pig protein (Hofmann *et al.*, 1979) rather than that of the minor A component of the cow protein used by Szebenyi *et al.* (1981). The native cow protein appears to be of the same length as that of the pig (Fullmer & Wasserman, 1980).

Results

Fluorescence spectra

Fig. 1(*a*) shows the baseline corrected emission spectrum of the Ca^{2+} form of intestinal CaBP, excited at 278nm. The maximum emission is found at 303nm; the relative quantum yield was $R_{Tyr} = 0.24$. The emission spectrum in Fig. 1(*b*) results from excitation of the protein at 263nm. The largest difference between these two spectra is the small shoulder in Fig. 1(*b*) arising from one or more phenylalanine residues in the protein, which fluoresce directly at about 282nm. Other phenylalanine residues, however, transfer their excitation energy via radiationless singlet–singlet transfer to the emitting tyrosine residue (Fig. 2*a*). Fig. 2(*a*) shows the baseline-corrected excitation spectrum at 303nm of the Ca^{2+} form of the protein, and Fig. 2(*c*), the model compound tyrosine. The excitation

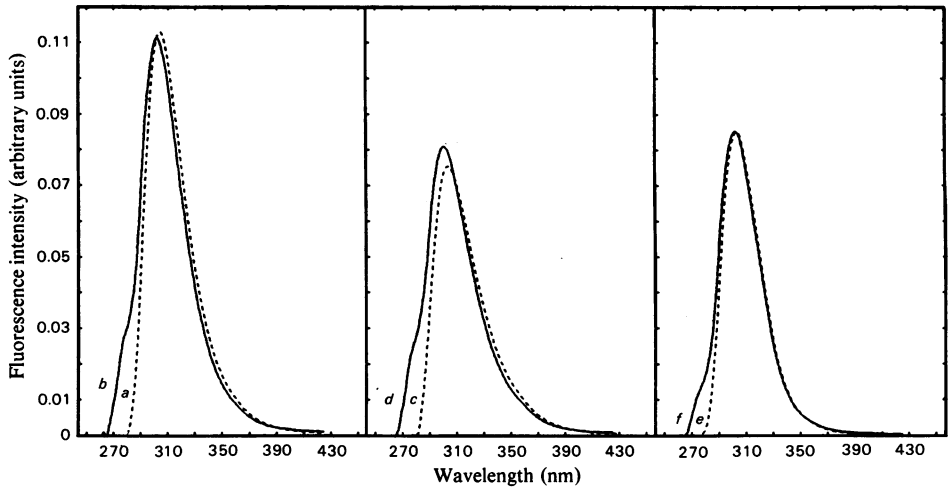


Fig. 1. *Fluorescence emission spectra of the Ca^{2+} and apo forms of CaBP of tyrosine and of a mixture of tyrosine and phenylalanine*

The Ca^{2+} form and apoprotein (68.4μM, A_{277} 0.115), tyrosine (37.3μM, $A_{274.6}$ 0.053) and tyrosine (26.1μM) plus phenylalanine (108.1μM) were in 10.0mM-Tris/HCl buffer, pH 8.0, also containing 150mM-NaCl. Spectra were recorded on cassette tapes at 40nm·min^{-1} with excitation and emission bandwidths of 4nm. Baseline spectra were recorded with solvent only. The cassette recordings were transferred to computer storage discs. Baseline spectra were subtracted from the sample spectra with a computer programme. (*a*) Ca^{2+} protein excited at 278nm; (*b*) Ca^{2+} protein excited at 263nm; (*c*) apoprotein excited at 278nm; (*d*) apoprotein excited at 263nm; (*e*) tyrosine excited at 263nm (this spectrum was normalized to the spectrum in *f* at 303nm); (*f*) a mixture of tyrosine and phenylalanine excited at 263nm.

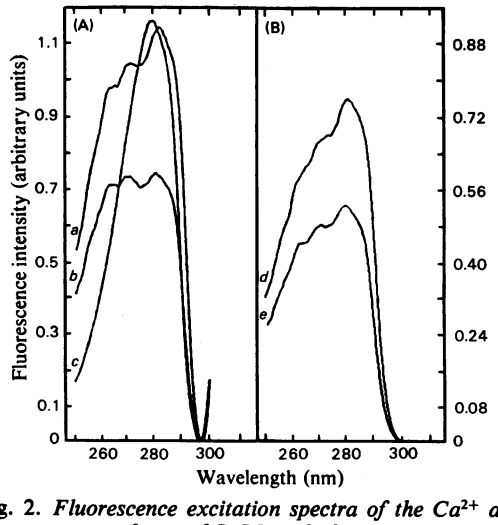


Fig. 2. *Fluorescence excitation spectra of the Ca^{2+} and apo forms of CaBP and of tyrosine*

(A) Conditions were the same as those described in the legend to Fig. 1, except that excitation spectra were collected at 20nm/min. Emission was monitored at 303nm. (*a*) Ca^{2+} protein; (*b*) apoprotein; (*c*) tyrosine (normalized to *a* at 280nm). (B) CaBP (52.3μM, A_{277} 0.088) excitation spectra were collected at 30nm/min under the same conditions as described for (A). Emission was monitored at 317nm. (*d*) Ca^{2+} protein; (*e*) apoprotein.

spectrum is similar to, but not identical with, its absorption spectrum and shows the phenylalanine fine structure very clearly. The latter is absent from the model compound.

Removing all the Ca^{2+} from the protein (see under 'Methods') quenches the tyrosine fluorescence to an R_{Tyr} of 0.16 (Fig. 1*c*), when the protein is excited at 278nm. The shoulder due to direct fluorescence of phenylalanine (Fig. 1*d*) is similar to that observed for the Ca^{2+} form of the protein. That this shoulder is not an experimental artifact is demonstrated by the emission spectra shown in Figs. 1(*e*) and 1(*f*). For these experiments, tyrosine alone and a mixture of tyrosine and phenylalanine were excited at 263nm under identical conditions. The tyrosine/phenylalanine mixture was made up to mimic approximately a situation in CaBP in which one phenylalanine residue fluoresces directly with a quantum yield equal to the free amino acid and one tyrosine residue fluoresces with an R_{Tyr} of 0.24. The spectrum of this mixture (Fig. 1*f*) shows a shoulder at around 282nm that is very similar to that observed in the emission spectra of the protein excited at 263nm (Figs. 1*b* and 1*d*).

Whereas the emission spectra of the two forms of CaBP are very similar (Figs. 1*a*–1*d*), the excitation spectra are distinctly different when monitored at 303nm (Figs. 2A and 2B). The ratio of the

fluorescence at 278nm to that at 268nm or 262nm is significantly higher in the Ca^{2+} form than in the apoprotein. However, when the emission is monitored at 317nm, the excitation spectra of both forms differ only in intensity (Figs. 2*d* and 2*e*).

Acid pH titrations

Protonation of the carboxylate groups of CaBP resulted in a large, reversible enhancement of the tyrosine fluorescence as shown in Fig. 3. The fluorescence titration curves of the Ca^{2+} and apo forms are nearly identical, except between pH5 and 8, where the apoprotein shows a greater degree of quenching. A Hill plot of the data of three experiments gave pK values of 4.25 ± 0.25 and 4.13 ± 0.20 respectively and slopes of 0.90 ± 0.13 and 0.93 ± 0.39 respectively for the Ca^{2+} and apo forms of the protein. Since the Hill slopes are close to unity, the fluorescence enhancement is not due to a co-operative denaturation, but rather to the protonation of one or more non-interacting carboxy groups. This interpretation is strengthened by the fact that protonation of CaBP does not result in any measurable change in its secondary structure as judged by the c.d. spectrum (not shown) in the peptide-bond absorption region.

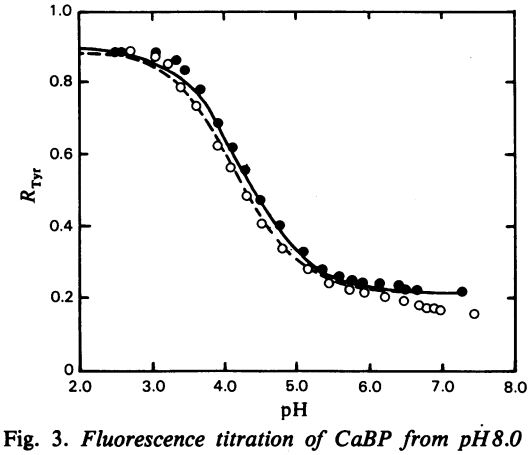


Fig. 3. *Fluorescence titration of CaBP from pH8.0 to pH2.5*

The Ca^{2+} form (●) and apoprotein (○) were titrated as described under 'Methods'. The protein was excited at 278nm with a 4nm band pass and the emission at 303nm was measured with a 2nm band pass. Protein concentration was 109.5μM (A_{277} 0.184) in 10.0mM-Tris/HCl buffer, pH8.0, also containing 150mM-NaCl. Hill plots of the data yielded pK values of 4.29 and 4.18 and Hill coefficients of 0.96 and 0.91 for the Ca^{2+} and apo forms respectively. From these, the theoretical curves were calculated (——, Ca^{2+} form; – – – –, apo form). The titrations were completely reversible (points not shown).

The titration curves of the c.d. band at 276nm show much more marked differences between the Ca^{2+} and apo forms of the protein (Fig. 4). As shown originally by Dorrington *et al.* (1974), Fig. 4 demonstrates that adding Ca^{2+} to the protein at pH8 increases the c.d. signal of the tyrosine from a small negative value to a large positive one. Protonation of the Ca^{2+} form (Fig. 4*a*) reversibly reduces the ellipticity with a pK of 4.05 and a Hill slope of 0.85 consistent with the fluorescence titration described above. However, protonation of the apoprotein results first in an increase in ellipticity (Fig. 4*b*) followed by a loss in signal intensity (Fig. 4*c*) at more acidic pH values. The pK values of these two overlapping transitions are not as readily determined as in the previous titrations. Theoretical curves have been calculated, assuming Hill coeffi-

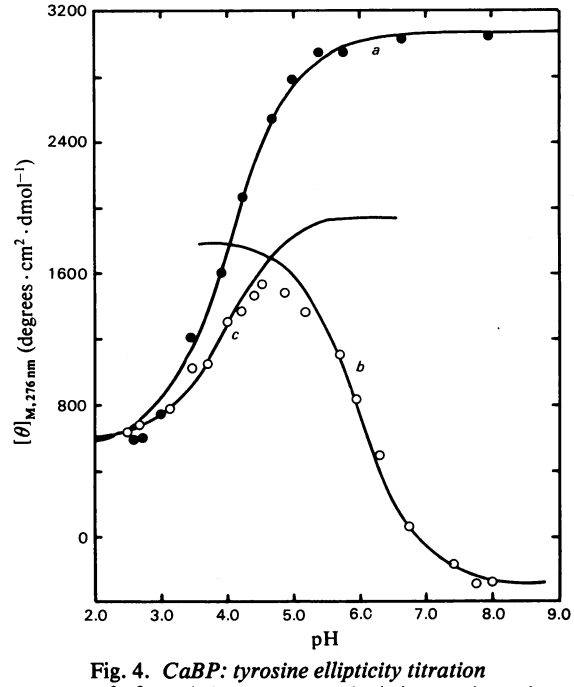


Fig. 4. *CaBP: tyrosine ellipticity titration*

The Ca^{2+} form (●) and apoprotein (○) were titrated to acidic pH as described under 'Methods'. C.d. spectra were recorded as described under 'Methods' and the molar ellipticity $[\theta]_{M,276nm}$ was calculated. Protein concentration was 113.6μM (A_{277} 0.191) in 10.0mM-Tris/HCl buffer, pH8.0, also containing 150mM-NaCl. A Hill plot of the data for the Ca^{2+} form gave a pK of 4.05 and a Hill coefficient of 0.85. From these, the theoretical curve (*a*) was calculated. The theoretical curves for the apoprotein data were derived by assuming Hill coefficients of unity and pK values of 4.0 and 6.0 for the descending (*c*) and ascending (*b*) data respectively. Both titrations were completely reversible (points not shown).

cients of unity and pK values of 4.0 and 6.0 for the descending and ascending curves respectively. These curves fit the data well, as shown in Figs. 4*b* and 4*c*. The data in Fig. 4 also demonstrate that the c.d. bands in both the Ca^{2+} form and the apoprotein are titrated to the same ellipticity value at low pH. Since the c.d. signal of the tyrosine is very sensitive to the presence of bound Ca^{2+} (Dorrington *et al.*, 1974), we assume that the titration of the two forms of the protein leads to the same final state at low pH, namely a Ca^{2+}-free form. This is confirmed in the c.d. spectra of the two forms at low pH (not shown), which are identical.

Alkaline pH titrations

If tyrosine is fully exposed to water, it will ionize with a pK between 9.6 and 10.0 (Tanford, 1962) with a red shift in the absorption maximum from 275 to 295nm (Donovan, 1973), loss of tyrosine fluorescence (Cowgill, 1976), and a general red shift of the tyrosine c.d. bands (Adler *et al.*, 1973).

From the small increase in absorption at 295nm as the protein is titrated to high pH shown in Fig. 5, it is evident that the pK of the tyrosine is unusually high; an extrapolated value of about 12.9 for the Ca^{2+} form can be calculated from the absorbance difference obtained at pH11.9 and the molar absorbance of ionized tyrosine (Donovan, 1973). A small difference apparently exists between the Ca^{2+} and apo forms, the apoprotein ionizing at slightly lower pH values than the Ca^{2+} form. C.d. spectroscopy confirms the unusually high pK of the tyrosine. At pH12.2, the spectrum (not shown) is unaltered except for a small decrease in ellipticity at 276nm and the appearance of a small shoulder between 290 and 310nm.

Unexpectedly, the tyrosine fluorescence of both forms of the protein is reversibly but not completely quenched at pH values that are below those where the absorbance begins to show tyrosine ionization (Fig. 6). The titration curves show pK values of 10.05 and about 10.4 respectively, for the Ca^{2+} and the apo forms. A Hill plot of the titration of the Ca^{2+} form gives a slope of 0.92. The fluorescence transition of the apoprotein is biphasic, being further quenched above pH11.5.

Discussion

The amino acid sequence of the intestinal CaBP from the cow is 87% identical with that of the pig protein (Hofmann *et al.*, 1979; Fullmer & Wasserman, 1981) and all six aromatic residues occupy identical positions. We assume that the three-dimensional structures are closely similar and that the spectral results presented in the present paper can be interpreted in terms of the structure of the cow protein (Szebenyi *et al.*, 1981).

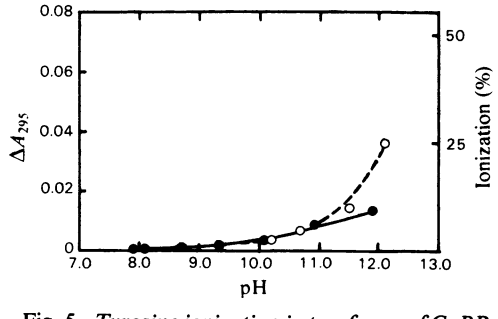


Fig. 5. *Tyrosine ionization in two forms of CaBP*
The Ca^{2+} form (●) and apoprotein (○) were titrated to basic pH as described under 'Methods'. The change in absorbance at 295nm was monitored with a Beckman DU-8 spectrophotometer. Protein concentrations were 60.7μM (A_{277} 0.102) in 10mM-Tris/HCl buffer, pH8.0, also containing 150mM-KCl. The ionization of the tyrosine hydroxy group (expressed as a percentage of complete ionization) was calculated assuming a change in molar absorbance for tyrosine ionization of 2330$M^{-1} \cdot cm^{-1}$ at 295nm (Donovan, 1973). The titrations were completely reversible below pH12 (points not shown).

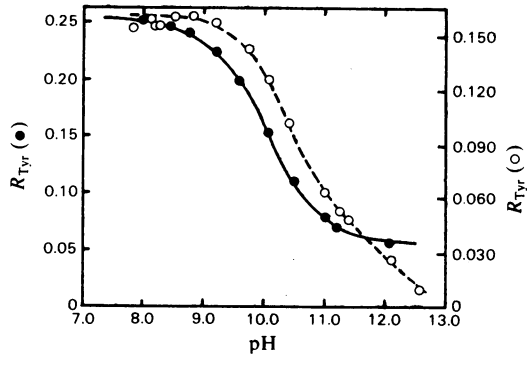


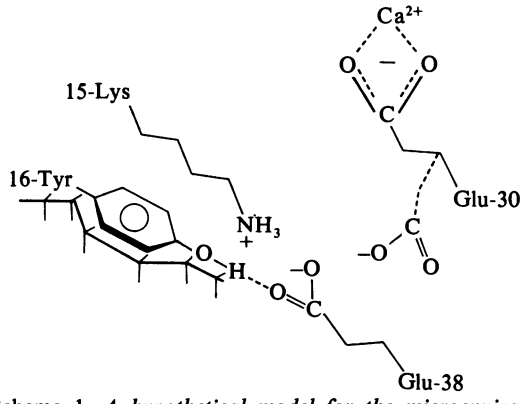
Fig. 6. *CaBP: tyrosine fluorescence quenching above pH8*
The Ca^{2+} form (●) and apoprotein (○) were titrated to basic pH as described under 'Methods'. The protein was excited at 278nm with a band pass of 4nm and the emission at 303nm monitored with a 5nm band pass. The protein concentration was 60.7μM (A_{277} 0.102) in 10.0mM-Tris/HCl buffer, pH8.0, also containing 150mM-KCl. A Hill plot of the Ca^{2+} protein data gave a pK of 10.05 and a Hill coefficient of 0.92. The theoretical curve (——) was derived from these parameters. The titrations were completely reversible below pH12 (points not shown).

Phenylalanine fluorescence and energy transfer

The lack of tryptophan and the presence of a single quenched tyrosine residue permits the observation of a small but definite phenylalanine fluorescence (Figs. 1*b* and 1*d*), which appears as a shoulder on the tyrosine emission peaks. Difference spectra (not shown) show that this phenylalanine emission has a maximum at 282 nm. The independent phenylalanine fluorescence is also apparent when one compares the excitation spectra in Fig. 2. When the emission is monitored at 303 nm there is a marked difference between the excitation spectra of the Ca^{2+} form and the apo form (Figs. 2*a* and 2*b*). In the apoprotein, the intensity is lower and the ratio of the intensity at 280 nm to the intensities at lower wavelengths is considerably higher than in the Ca^{2+} form. However, there is no difference in the shape of the spectra between the two forms when the emission is monitored at 317 nm (Figs. 2*d* and 2*e*), although the fluorescence intensity is lower in the apo form. The excitation spectra monitored at 303 nm include contributions from direct phenylalanine emission. Although we cannot determine the number of directly emitting phenylalanine residues, the fine structure below 270 nm in the excitation spectra monitored at 317 nm shows that some of the five phenylalanine residues transfer their excitation energy to the tyrosine. Jones *et al.* (1980) reported complete resonance energy transfer ($100 \pm 10\%$) from all five phenylalanine residues to the tyrosine in cow intestinal CaBP. However, our results are in agreement with the three-dimensional structure of the cow protein (Szebenyi *et al.*, 1981). This shows that three phenylalanine residues (phenylalanine-13, -39 and -69) are within 1.1 nm of the tyrosine and are thus close enough for a direct transfer. The other two are more remote from the tyrosine and it is probably one or both of these (phenylalanine-53 and -66) that fluoresce independently.

Tyrosine fluorescence

Szebenyi *et al.* (1981) report that tyrosine-16 lies in a very shallow depression on the surface of the protein formed by isoleucine-12, leucine-33, leucine-34, threonine-37 and glutamate-38 (in the pig protein leucine-33 is replaced by glutamine-33 and threonine-37 by alanine-37), with a hydrogen bond between tyrosine-16 and glutamate-38 being 'probable'. The tyrosine is positioned in α-helix I, which spans residues 5–17. Residue 15 is a lysine molecule. An α-helical model of the sequence containing lysine-15 and tyrosine-16 shows that the positive charge of the ε-amino group can be brought into close proximity with the tyrosine hydroxy group. Model building based on the backbone folding pattern suggests that glutamate-30, one of the Ca^{2+}-co-ordinating ligands, may be close enough to influence the tyrosine in the absence of Ca^{2+}.



Scheme 1. *A hypothetical model for the microenvironment of tyrosine-16*

This model summarizes the possible interactions between tyrosine-16 and the side chains of three nearby amino acid residues in pig intestinal CaBP. The evidence for these interactions comes from the spectroscopic results for the pig protein and a tentative interpretation of the three-dimensional structure of the cow protein as proposed by Szebenyi *et al.* (1981). A glutamate residue (possibly glutamate-30) is shown in two positions representing possible different conformations in the Ca^{2+} form (——) and the apoprotein form (· · · ·).

Scheme 1 shows an approximate representation of the groups around tyrosine-16, as a guide to the interpretation of the data in the present paper.

The tyrosine fluorescence of the Ca^{2+} form of the pig protein is quenched to an R_{Tyr} of 0.24, presumably by the interaction between tyrosine-16 and glutamate-38, suggested by Szebenyi *et al.* (1981).

In the Ca^{2+}-free form the tyrosine fluorescence is further quenched to an R_{Tyr} of 0.16. Ca^{2+} removal does not result in any significant rearrangement of the secondary structure of the pig protein as judged by c.d. studies (Dorrington *et al.*, 1974), although some changes in the tertiary structure of the cow protein have been observed with n.m.r. spectroscopy (Levine *et al.*, 1977). Ca^{2+} removal probably increases the number of negative charges in the vicinity of tyrosine-16 and may specifically bring glutamate-30 closer to tyrosine-16, as suggested in Scheme 1.

Tyrosine ionization

In spite of the fact that tyrosine-16 apparently lies on the surface of the molecule (Szebenyi *et al.*, 1981), its hydroxy group has a pK of about 12.9, i.e., three pH units higher than that of an unhindered group. The titration of an n.m.r. spectrum also confirms the high pK value (J. Shelling, J. D. J. O'Neil,

T. Hofmann & B. Sykes, unpublished work). The high pK may be due to two factors. (*a*) The hydrophobic depression in which tyrosine-16 lies provides an unfavourable environment for an unhindered ionization. (*b*) the interaction with glutamate-38 stabilizes the proton of the tyrosine hydroxy group. In fact, it is unlikely that the tyrosine can ionize without disruption of the protein structure. If the titration is carried beyond pH 12, the protein denatures and the titration is no longer reversible.

Since the fluorescence of the ionized form of tyrosine at 303 nm is completely quenched (White, 1959), we anticipated from the u.v.-absorption experiment that the fluorescence titration curve in the alkaline region would be similar to the absorption curve (Fig. 5) and be quenched only above pH 12. Unexpectedly, as Fig. 6 shows, the tyrosine fluorescence of the Ca^{2+} form is quenched with a pK of 10.05 and a Hill coefficient of 0.92. This is clearly not due to tyrosine ionization but must be caused by ionization of one or more lysine residues in the protein, which usually have a pK of 10.0–10.2 (Cantor & Schimmel, 1980). Model studies by Weber & Rosenheck (1964) and Feitelson (1964) show that unionized free amino groups quench tyrosine fluorescence by collisional proton transfer from the tyrosine. In Scheme 1, we propose that the deprotonation of lysine-15 may be responsible for this quenching effect via a direct proton transfer. The fluorescence of the apoprotein is quenched in two stages (Fig. 6). The first stage is similar to the quenching of the Ca^{2+} form, but with a higher pK, probably due to the increase in the negative charges in the proximity of lysine-15. Above pH 11.5 (the second stage), quenching is probably the result of tyrosine ionization. This agrees with the absorbance changes (Fig. 5) which indicate a higher degree of ionization of the apo form than of the Ca^{2+} form at any pH above 11. The Ca^{2+} form is clearly more stable than the apo form and, as we have proposed above, the ionization of the tyrosine cannot occur without disruption of the protein structure.

Fluorescence and c.d. titration to low pH

The fluorescence (Fig. 3) and c.d. (Fig. 4*a*) titration curves of the Ca^{2+} form to low pH are most easily explained by postulating that the major cause for the alleviation of the quenching and the decrease in the ellipticity band respectively is the protonation of the glutamate-38 anion with other carboxylates exerting less influence, if any. The fluorescence and c.d. titration curves of the apo form show two stages, the first with a pK of around 6.0, which can be ascribed to at least one of the Ca^{2+}-binding carboxylate groups. The second stage shows a pK of 4.17 (fluorescence) or 4.0 (c.d.). As in the Ca^{2+} form the increased quantum yield is probably due to the protonation of the glutamate-38 anion. During this titration, both calcium ions are removed from the Ca^{2+} form so that at the lower end of the titration curves the fluorescence yields and ellipticity values of the 'Ca^{2+} form' and the apoprotein are the same.

C.d. spectra of the peptide bond absorption region at low pH (not shown) are identical with those obtained at neutral pH and show that no significant changes in secondary structure have occurred during the low-pH titrations. Below pH 3 (Fig. 3) the R_{Tyr} is 0.85, a value observed for tyrosine in an α-helix (Cowgill, 1976).

Circular dichroism of tyrosine-16

The mechanism whereby Ca^{2+} binding and carboxylate protonation affect the tyrosine c.d. spectrum is not clear. It is not obvious why the removal of Ca^{2+} should result in the complete abolition of the strong 276 nm ellipticity band, when the effect on fluorescence is only a slight decrease in quantum yield that can be ascribed to the increase in negative charges near the tyrosine. The alkaline titration also shows that the high pK of the tyrosine hydroxy group is lowered only slightly in the apoprotein, suggesting that the hydrophobic interaction of tyrosine and its interaction with glutamate-38 are only little perturbed.

On the other hand, Szebenyi *et al.* (1981) pointed out that removal of Ca^{2+} from crystalline CaBP breaks up the crystals. Furthermore, the apoprotein has so far not been obtained in a crystallized form. This suggests that the apoprotein has a looser structure and presumably altered positions of surface amino acid residues. The most likely explanation of the effect of removing Ca^{2+} on the ellipticity band is a direct effect of at least one Ca^{2+}-binding glutamate ion. The evidence for this lies in the c.d. titration curve (Fig. 4*b*), which shows that protonation of the high-pK Ca^{2+}-binding carboxylate ions restores the tyrosine ellipticity band, at least in part. Protonation of the carboxylate of glutamate-38 specifically and most of the other carboxylates of the protein in general leads to a reduction, but not a complete abolition, of the ellipticity band (Fig. 4) in both the Ca^{2+} form and the apoprotein.

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