Binding of a spin-labelled chlorpromazine analogue to calmodulin

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The binding of a spin-labelled derivative of chlorpromazine to calmodulin was investigated by e.s.r. spectrometry. The completion of the spectroscopic changes requires the presence of 4 Ca^{2+} ions per calmodulin molecule. The influences of various physicochemical factors (pH, ionic strength) are discussed in relation to the nature (hydrophobic and polar) of the interactions that hold the drug–calmodulin complex together.

The interaction of calmodulin with its target enzymes requires the exposure of a lipophilic domain (Means, 1981). The binding of Ca^{2+} to the high-affinity binding sites of calmodulin induces a conformational change that displays this hydrophobic domain. Meanwhile calmodulin becomes able to interact with a whole variety of hydrophobic molecules [naphthalene derivatives (Laporte *et al.*, 1980), phenothiazine derivatives (Levin & Weiss, 1977) and chloronaphthalenesulphonamide derivatives (Tanaka *et al.*, 1982)]. These molecules may have pharmacological relevance if they are able to block the activation of the target enzymes by Ca^{2+} –calmodulin (Cheung, 1980).

The binding of phenothiazine derivatives to the lipophilic domain of calmodulin (Roufogalis *et al.*, 1983) is well documented. It displays a low specificity: various isomers for the chlorine position or with different side chains bind equally well to calmodulin (Roufogalis, 1981). Nevertheless a combination of hydrophobic and polar interactions (Gariépy & Hodges, 1983) provides K_d values all around 10^{-5} – 10^{-6} M for the phenothiazine–calmodulin complex.

To probe the phenothiazine-binding site of calmodulin, a spin-labelled derivative of chlorpromazine (CPZSL) has now been used.

Closely related spin probes have already been used in other fields: DNA structure (Bobst, 1979) or membranes (Manian *et al.*, 1974).

In the present paper it is shown that the spin-labelled reporter group has a high sensitivity to various effectors in the environment of the drug-

binding site, and this gives additional information to that obtained in previous fluorescence studies.

Materials and methods

CPZSL was prepared from norchlorpromazine [2-chloro-10-(3-aminopropyl)phenothiazine (generously provided by Dr. F. Letierrier, C.R.S.S.A., Clamart, France)] and the acid chloride of 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-*N*-oxyl (purchased from Eastman). The amidification of the amino group at the end of the side chain of norchlorpromazine by the acid chloride was conducted under the conditions previously described (Manian *et al.*, 1974). CPZSL (see Fig. 1) was purified successively by column chromatography on silica gel with diethyl ether as solvent and by t.l.c. on silica gel with chloroform/methanol (9:1, v/v) as solvent.

Calmodulin was isolated from ram testis and purified by taking advantage of its Ca^{2+} -dependent binding to phenyl-Sepharose (Pharmacia) as described in Autric *et al.* (1980).

The concentrations of calmodulin (CAM) and of CPZSL were measured by spectrophotometry at 227 nm (ϵ_{CAM} $3300 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and at 257 nm (ϵ_{CPZSL} $43000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) respectively.

Contaminating metal ions were removed from buffer solutions by passage through a Chelex 100 (Bio-Rad Laboratories) column as described in Haiech *et al.*, 1979). Removal of Ca^{2+} from calmodulin was achieved by trichloroacetic acid precipitation as described by Haiech *et al.* (1981). E.s.r. spectra were recorded with a Bruker ER 200 spectrometer thermostatically controlled with the variable-temperature unit. The field was modulated at 100 kHz by an alternative field of 0.16 mT (1.6 G).

Abbreviations used: CPZ, chlorpromazine; CPZSL, spin-labelled chlorpromazine; $H(-1)$, high-field peak amplitude; $H(0)$, mid-field peak amplitude.

The microwave (9.65 GHz) irradiation was kept under 20 mW to prevent saturation. The sample volume was 500 μ l. The signal was digitized and computerized by using an Apple II in-line with the spectrometer.

Results

Binding of CPZSL to calmodulin

Clear differences (Fig. 1) are recorded in the resonance spectra of CPZSL when calmodulin is added in a medium containing Ca^{2+} . The resonance lineshape changes from a rapid isotropic motion [$H(-1)/H(0) = 0.85$] with a rotational correlation time around 0.1 ns to a weakly immobilized motion [$H(-1)/H(0) < 0.8$]. These modifications are close to the spectra observed for CPZSL interacting with erythrocyte membranes (Manian *et al.*, 1974), with the difference that the degree of immobilization and/or the anisotropy of the motion is less pronounced.

Consequently no strongly immobilized peak (peak S) corresponding to a large hyperfine splitting can be resolved. Such a peak S was found for the binding of the probe to erythrocyte membranes and for the binding to albumin (results not shown). With a calmodulin reversibility of the spectral modifications in the presence of EDTA is complete (Fig. 1).

Determination of the dissociation constant of the CPZSL-calmodulin complex

The plot of the ratio $H(-1)/H(0)$ versus the concentration of calmodulin (Fig. 2) displays a change from its upper limit (0.85 in the absence of calmodulin) down to 0.5 (the lowest observable value when the excess of calmodulin over CPZSL is at maximum). Moreover the binding curves are shifted upward when the concentration of CPZSL is gradually increased from 0.5 to 2 μ M.

The variation of the ratio $H(-1)/H(0)$ becomes independent of the CPZSL concentration when it is higher than 2 μ M. Consequently we assume that the K_d of the CPZSL-calmodulin complex is below 2 μ M. But the spectra obtained for these very low (<1 μ M) concentrations of CPZSL offer a poor reliability, and this prevents the determination of a K_d value with sufficient accuracy.

Ca^{2+} -dependency of the spectrum

Fig. 3 shows the decrease in $H(-1)/H(0)$ when the Ca^{2+} concentration is increased. This variation is independent on the CPZSL concentration (5 or 15 μ M) provided that the probe is not present in excess in proportion to the protein. The variation of $H(-1)/H(0)$ is gradual up to 4 Ca^{2+} -binding sites.

Fig. 1. Structure of the spin-labelled chlorpromazine (CPZSL) and its binding to calmodulin monitored by e.s.r. E.s.r. spectra are recorded (a) in 0.2 mM- CaCl_2 /100 mM-Tris/HCl buffer, pH 7.4, (b) as in (a) + 50 μ M-calmodulin, and (c) as in (b) + EGTA. High-field and mid-field peaks are respectively defined as $H(-1)$ and $H(0)$.

Influence of the ionic strength on the CPZSL-calmodulin interaction

Fig. 4 displays the relative variation of $H(-1)/H(0)$ when NaCl is added to calmodulin recalcified to various degrees of Ca^{2+} occupancy (1, 2 and 4 Ca^{2+} ions per calmodulin molecule). It is clear that the effect of the ionic strength becomes apparent

Fig. 2. Binding of CPZSL to calmodulin plotted as a function of calmodulin concentration

To various concentrations of CPZSL ($\square$, 0.5 μM ; $\bullet$, 1 μM ; $\blacktriangledown$, 2 μM ; $\blacksquare$, 5 μM ; $\circ$, 7.5 μM ; $\blacktriangle$, 10 μM) in 0.1 mM- CaCl_2 /10 mM-Tris/HCl buffer, pH 7.4, the protein was added gradually in highly concentrated solution (final variation of sample volume is kept under 10%).

Fig. 3. Binding of CPZSL to calmodulin plotted as a function of Ca^{2+} concentration

To Ca^{2+} -free calmodulin (15 μM) in the presence of CPZSL ($\bullet$, 5 μM ; $\triangle$, 15 μM) in decalcified 10 mM-Tris/HCl buffer, pH 7.4, Ca^{2+} is added gradually.

Fig. 4. Influence of NaCl on the binding of CPZSL (15 μM) to calmodulin in the presence of various concentrations of Ca^{2+}

Calmodulin (15 μM) is recalcified with 1 ($\circ$), 2 ($\blacktriangle$) or 4 ($\bullet$) mol of Ca^{2+} per mol of calmodulin and the relative variation of $H(-1)/H(0)$ as a percentage, is recorded as a function of the concentration of NaCl in the medium (10 mM-Tris/HCl buffer, pH 7.4).

only after that the two high-affinity Ca^{2+} -binding sites have been saturated.

Influence of the pH on the CPZSL-calmodulin interaction

Fig. 5 displays the variation of $H(-1)/H(0)$ when the pH of a sodium barbital-acetate buffer (Michaelis, 1931) is varied from 3 to 10. The sharp decrease around pH 4.5 is related to the alteration of the protein at the pH_i , as previously described by Dedman *et al.* (1977). But it is worth noticing that between 4.5 and 10 a continuous decrease of $H(-1)/H(0)$ is observed.

Discussion

Interpretation of the lineshape of the spectrum

The reporter group of the bound CPZSL might *a priori* tumble with three different frequencies: (1) the rotational diffusion rate of the protein itself, (2) the intramolecular segmental motion of the acyl side chain between the phenothiazine ring and the pyrrolinoxyl ring, and (3) the motion of the phenothiazine ring relative to the calmodulin.

Item (3) can be considered to be beyond the e.s.r. time scale because it is likely that this type of motion is sufficiently slow and anisotropic to generate a separate peak S with a large hyperfine splitting. Such a peak ('Strongly immobilized') is,

Fig. 5. Influence of pH on the binding of CPZSL. Calmodulin (20 μM) and CPZSL (20 μM) are allowed to bind in a medium containing 100 μM - Ca^{2+} . The pH of the barbital/acetate buffer (30mM) is varied between 10 and 3 by addition of HCl.

however, observable when the probe is bound to albumin (D. Rainteau, unpublished work). The binding of CPZSL to calmodulin is followed by a change of spectrum from a fast tumbling rate to a weakly immobilized motion. This change has been mimicked by decreasing the temperature of a sample of CPZSL in white oil (Primol 242) from 327K to 295K. This corresponds to an increase in viscosity from approx. 2.8 Pa·s (28cP) to 16.5 Pa·s (165cP), which in turn corresponds to an increase in the rotational correlation time ($\tau_R \times 5.8$).

The correlation time determined from the spectra by the procedure of Freed & Fraenkel (1963) displays also an increase in the tumbling rate from 0.13 to 0.7 ns ($\tau_R \times 5.4$) when CPZSL is allowed to interact with calmodulin. This may be compared with the slight increase (+6%) of τ_R for covalently spin-labelled calmodulin (Hewgley & Puett, 1980) in the presence of Ca^{2+} . This change in τ_R is consistent with a compaction of the protein on binding of 4–5 mol of Ca^{2+} per mol of calmodulin (Siegel *et al.*, 1983).

Under these conditions it is obvious that the CPZSL probe offers a high sensitivity for the monitoring of conformational changes of this protein, and particularly of the hydrophobic surface, where drugs and target enzymes are believed to interact.

E.s.r. provides qualitative information on the drug-calmodulin complex

The e.s.r. signal can originate from the free as well as from the bound probe, in contrast with the fluorescence method, where the free probe in water has such a low quantum yield that the final fluorescence intensity can be directly related to the amount of bound probe.

Another feature of e.s.r. is that not only can the spectrum be altered by the fraction bound/free probe but also it can be qualitatively modified by the degree of immobilization of the bound probe. In the present paper we wish to emphasize that both mechanisms are required to explain all the spectral modifications recorded.

The fluorescence method has established that the exposure of the hydrophobic area of calmodulin is terminated when 2 Ca^{2+} ions are bound per calmodulin molecule (Klee & Vanaman, 1982). The e.s.r. spectrum, on the other hand, is modified in the presence of up to 4 Ca^{2+} ions per calmodulin molecule. This is consistent with results from n.m.r. (Seamon, 1980), where the resonances of various acyl protons have been shown to be influenced after the two high-affinity Ca^{2+} -binding sites have already been filled. This is also consistent with the e.s.r. results obtained with covalently labelled calmodulin (Siegel *et al.*, 1983), where 4–5 Ca^{2+} ions per calmodulin molecule are required to immobilize the spin probe maximally.

We assume here that the decrease of $H(-1)/H(0)$ follows the co-operative binding of Ca^{2+} to the two first high-affinity sites of calmodulin and is essentially related to the binding of the probe to the lipophilic area. The difference between the fluorescence and the e.s.r. study lies on the influence of the two lower-affinity Ca^{2+} -binding sites.

A two-step transition has also been assumed after proton-n.m.r. studies (Seamon, 1981). The first change occurs on addition of 0–2 mol of Ca^{2+} per mol of calmodulin and exhibits a slow exchange behaviour in agreement with a slow conformational change. The second set of spectral changes is observed on addition of 2–4 mol of Ca^{2+} per mol of protein, and the associated transition is fast on the n.m.r. time scale. This could then be explained by the setting of weak polar interactions between the drug and calmodulin. The following part of the discussion provide evidence that supports this assumption (1). The hyperfine splitting of bound CPZSL remains around 1.6mT (16G) at 20°C. This supports strongly the suggestion that the reporter group is located in an high-polarity environment, probably distinct from the lipophilic area, where the phenothiazine ring is intercalated. (2). In this polar environment the motion of the nitroxide group is sensitive to ionic strength only if there are more than 2 Ca^{2+} ions per calmodulin

molecule (see Fig. 4). Under these conditions we assume that the ionic strength is able to counteract the polar interactions induced by the occupation of the two lower-affinity Ca^{2+} -binding sites (3). The decrease of $H(-1)/H(0)$ when the pH is increased beyond 5 can be also interpreted as an increased polarization of interacting groups inside the drug-calmodulin complex.

Finally we assume, in accordance with the model proposed by Gariépy & Hodges (1983), that the effect of the occupancy of the two lower-affinity Ca^{2+} -binding sites is to put in place one of the numerous polar groups acidic (Glu-82, -83 and -84) or basic (Arg-86 and -90). This will give the reporter group the opportunity of a polar interaction in the vicinity of the lipophilic domain where the phenothiazine ring is located (Phe-89 and -92).

References

- Autric, F., Ferraz, C., Kilhoffer, M. C., Cavadore, J. C. & Demaille, J. G. (1980) *Biochim. Biophys. Acta* **631**, 139–147
- Bobst, A. M. (1979) *Spin Labeling* **2**, 291–342
- Cheung, W. Y. (1980) *Science* **207**, 19–27
- Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D. & Means, A. R. (1977) *J. Biol. Chem.* **252**, 8415–8422
- Freed, J. H. & Fraenkel, G. K. (1963) *J. Chem. Phys.* **39**, 326–331
- Gariépy, J. & Hodges, R. S. (1983) *Biochemistry* **22**, 1586–1594
- Haiech, J., Derancourt, J., Pechere, J. F. & Demaille, J. G. (1979) *Biochemistry* **18**, 2752–2759
- Haiech, J., Klee, C. B. & Demaille, J. G. (1981) *Biochemistry* **20**, 3890–3897
- Hewgley, P. B. & Puett, D. (1980) *Ann. N.Y. Acad. Sci.* **356**, 20–31
- Klee, C. B. & Vanaman, T. C. (1982) *Adv. Protein Chem.* **35**, 213–321
- LaPorte, D. C., Wierman, B. M. & Storm, D. R. (1980) *Biochemistry* **19**, 3814–3819
- Levin, R. M. & Weiss, B. (1977) *Mol. Pharmacol.* **13**, 690–697
- Manian, A., Piette, L. H., Holland, D., Grover, T. & Leterrier, F. (1974) in *The Phenothiazines and Structurally Related Drugs* (Forrest, I. S., Carr, C. J. & Usdin, E., eds.), pp. 149–161, Raven Press, New York
- Means, A. R. (1981) *Recent Prog. Horm. Res.* **37**, 333–343
- Michaelis, L. (1931) *Biochem. Z.* **234**, 139–142
- Roufogalis, B. D. (1981) *Biochem. Biophys. Res. Commun.* **98**, 607–613
- Roufogalis, B. D., Minocherhomjee, A. M. & Al-Jobore, A. (1983) *Can. J. Biochem.* **61**, 927–933
- Seamon, K. B. (1980) *Biochemistry* **19**, 207–215
- Siegel, N., Coughlin, R. & Hang, A. (1983) *Biochem. Biophys. Res. Commun.* **115**, 512–517
- Tanaka, T., Ohmura, T. & Hidaka, H. (1982) *Mol. Pharmacol.* **22**, 403–407