

TIME-RESOLVED TRYPTOPHAN FLUORESCENCE OF FRAGMENT 1-86 OF FACTOR X AND THE INFLUENCE OF MEMBRANE BINDING

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Membrane binding of the N-terminus of the prothrombinase reaction enzyme, fragment 1-86 of factor X (FX F1-86), to phospholipid vesicles was studied using time-resolved fluorescence spectroscopy of the tryptophan residue Trp41 which is located in the membrane binding part of this protein (Gla domain). Fluorescence lifetimes were determined by the time-correlated single photon counting technique using synchrotron radiation as the excitation source. Different negatively charged lipid vesicles containing either phosphatidylserine or phosphatidylglycerol mixed with phosphatidylcholine were investigated. Pure synthetic lipids (dilauroyl and dioleoyl lipids) as well as natural lipids (from egg yolk or bovine brain) were used in membrane binding studies. Multiexponential data analysis supports the results already found for FX F1-86 in the presence of calcium ions. For all lipid systems we obtained decay times in the range of 0.2, 0.6, 2.6, 5.6 ns. Moreover, a long-lifetime component with minor contribution to the decay amplitude (<5%) was found. The fluorescence lifetimes do not show any wavelength dependence. There was no change in the fluorescence decay times upon membrane binding, which might indicate that a lipid-specific conformational change in the Gla domain of factor X does not take place upon membrane binding.

Keywords: Protein fluorescence; Factor Xa; Gla domain; Blood coagulation; Lipid-specificity; Prothrombinase; EGF-like domain; Synchrotron radiation; Enzymes.

The catalytic activity of the prothrombinase complex depends strongly on the lipid composition of the membrane and it is known that some acid lipids increase the turnover number of prothrombin tremendously. In the

present case phosphatidylserine appears to be the most efficient. In comparison, much lower concentrations of phosphatidylserine (PS)⁺ than phosphatidylglycerol (PG) are necessary for obtaining the same high rate in thrombin formation¹. On the other hand, the prothrombinase reaction enzyme, factor Xa, or the substrate, prothrombin, show similar binding affinities to differently composed membranes (*e.g.* PG- versus PS-containing). The molecular background of the lipid specificity is still unknown, which gives the motivation for characterising the interaction between the proteins involved, calcium ions, and negatively charged phospholipid surfaces.

In a recent work² we have shown that the N-terminal Gla domains of both factor Xa and prothrombin show nearly the same fluorescence spectroscopic properties in the absence of calcium ions and that those properties respond to calcium addition in a very similar manner. In both proteins, the calcium-induced conformational change in the Gla domain leads to equilibrium of a ground-state complex formed by the Gla tryptophan and a disulfide bridge located in the vicinity of the individual tryptophan residue on one hand and the non-complexed tryptophan on the other. The interaction between the aromatic π system and the disulfide bridge leads to a fluorescence decrease of about 70%. The calcium-induced conformational change in Gla domains is essential for membrane binding and has been investigated by other experimental methods, such as circular dichroism, differential scanning calorimetry as well as Fourier transform spectroscopy^{3,4}. The calcium-induced changes in the parameters determined by those methods are substantially smaller than the above decrease in the tryptophan fluorescence intensities. Moreover, experimental work and structure data indicate direct involvement of such a tryptophan disulfide adduct in the binding of Gla domains to phospholipid membranes⁵.

Figure 1 shows the structure of fragment 1-86 of factor X. It contains the N-terminal Gla domain followed by an epidermal growth factor-like domain, with a single tryptophan residue Trp41 located in the Gla domain. In contrast, bovine prothrombin fragment 1 (BF1) consists of 156 amino acids

+ Abbreviations: Apo-, without calcium; BF1, bovine prothrombin fragment 1; FX F1-86, fragment 1-86 of factor X; Gla domain, domain containing γ -carboxyglutamic acid residues; EGF, epidermal growth factor; DO, dioleoyl; DL, dilauroyl; eyPC, egg yolk phosphatidylcholine; eyPG, egg yolk phosphatidylglycerol; bbPS, bovine brain phosphatidylserine; MEM, maximum entropy method; Trp, tryptophan; PS, phosphatidylserine; PG, phosphatidylglycerol; LUV, large unilamellar vesicles; PC, phosphatidylcholine.

which build the Gla domain followed by a “kringle” domain. Altogether three tryptophan residues are located in BF1: one residue in the Gla domain and two residues in the kringle domain. When bound to PS-containing vesicles, the fluorescence lifetimes of BF1 remain unchanged, but binding to PG-containing vesicles leads to an increase the lifetimes of the components that were assigned to Trp42 residue located in Gla domain, possibly indicating a PG-specific conformational change³. For example, the decay time of the 5.3 ns component of Trp42 in Ca-BF1 (BF1 in the presence of calcium ions) increases from 5.3 to 7.5 ns. The hypothesis of a PG-specific conformational change in the Gla domain is supported by a FTIR study of BF1 when bound to PS-containing as well as to PG-containing membranes⁴. Accepting that the differences in the spectra (see ref.⁴, Fig. 7) of the amide I' region of apo- and Ca-BF1 can be interpreted in terms of the above described calcium-induced conformational change in the Gla domain, it is certainly tempting to conclude that PG-containing membranes induce a conformational change in BF1, whereas binding to PS-containing surfaces does not alter the BF1 conformation.

It is of interest to know whether the Gla domain of fragment 1-86 of factor X (FX F1-86) shows a similar membrane binding behaviour as BF1 and whether the fluorescence characteristics are altered again in a lipid-specific manner. Therefore we characterised the fluorescence decays of FX F1-86 bound to large unilamellar vesicles (LUV) of different phospholipid compositions.

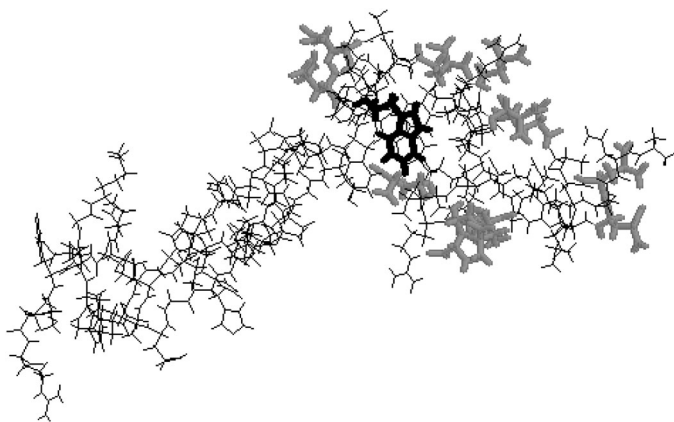


FIG. 1

Two-dimensional NMR structure of fragment 1-86 of factor X (Brookhaven protein data bank). Trp41 (black bold) is located in the Gla domain, which contains Gla residues (hatched grey)

EXPERIMENTAL

Proteins

Bovine factor X fragment 1-86 (FX F1-86) was obtained by tryptic digestion of factor X and purified chromatographically on a Mono-Q column (Pharmacia) by the method of Person *et al.*⁶ Factor X itself was isolated according to the procedure of Esnouf *et al.*⁷ The final concentration of fragment 1-86 in each experiment was 10 μM in Tris-buffered saline (0.05 M Tris, 0.1 M NaCl, pH 7.5) containing 7 mM CaCl_2 and 1 mM phospholipid.

Phospholipid Vesicles

Experiments were carried out with different lipid systems. For ensure that at least 85% of FX F1-86 was bound to the lipid membrane, the lipid concentration was determined using the following equation⁸:

$$P_L = \frac{P_b}{P_f} \frac{3}{2} \frac{N}{n} (K_d + P_f).$$

P_L is the total phospholipid concentration; P_b and P_f are the concentrations of bound and free protein, respectively. N/n indicates how many lipid molecules are bound per protein molecule and K_d is the dissociation constant of the protein/lipid complex. The determined K_d and N/n values appeared to be in the same range for all investigated lipid systems containing either 25% PS or 40% PG lipids (or equivalent synthetic lipids)³. For the determined values of K_d of about 0.5 μM and the determined values of N/n of about 40, 1 mM lipid is sufficient to bind more than 85% of all protein molecules to the membrane in a 10 μM solution^{3,8}. Thus the used lipid and protein concentrations ensured that we record fluorescence decays of bound protein to large extent.

Large unilamellar vesicles (LUV) were prepared by extrusion of a suspension of multilamellar vesicles classically obtained by hydration of a dried lipid film and subsequent vigorous vortexing. The extrusion was performed through polycarbonate filters (Nucleopore) using a Lipex Biomembranes extruder. The size of the filter pores was first 0.2 μm (7 passages) and thereafter 0.1 μm (10 passages) in order to obtain vesicles 0.11–0.12 μm in diameter. The lipids were mixed in the ratios 75:25 and 60:40 in the case of PC–PS and PC–PG mixtures, respectively. The phospholipids used (all from Sigma) were either natural (egg yolk PC (eyPC) and egg yolk PG (eyPG), bovine brain PS (bbPS)) or synthetic (dilauroyl (DL) or dioleoyl (DO) phospholipids).

Time-Resolved Fluorescence Measurements and Data Analysis

The fluorescence decay curves of FX F1-86 bound to LUV of different compositions were determined on the SA1 beam line at the LURE (Laboratoire pour l'Utilisation du Rayonnement Electromagnétique) in Orsay (France) using the single photon counting technique. For detailed information about the optical and electronic parts, see ref.⁹ The radiation of Super-ACO synchrotron was used for pulsed excitation with approximately 650 ps full width at half-maximum at a frequency of 8.3 MHz. The excitation wavelength at 295 nm was vertically polarised and the emission was detected, after passing a "magic angle" polariser, with a microchannel plate photomultiplier. The fluorescence decays were detected in the range 315–405 nm with 7.5 nm steps. The time-scale was 24.93 ps per channel on 2048 channels and up to 50 000 fluorescent photons were collected in the peak.

Experiments were carried out with 10 μM factor X fragment 1-86 of factor X in Tris-buffered saline containing 1 mM phospholipids and 7 mM CaCl_2 at 20 °C. Reference measurements were carried out with FX F1-86 together with pure PC vesicles (no membrane binding) as well as on pure PC vesicles alone.

Basic data analysis was carried out using a non-linear least-squares iterative reconvolution procedure based on the Marquardt–Levenberg algorithm^{10,11}. In addition, data were analysed by the maximum entropy method (MEM). This method uses distribution of lifetimes with no apriori assumption about the shape of the distribution¹². All the presented data were obtained by the non-linear least-squares iterative reconvolution procedure.

RESULTS

For FX F1-86, either in the presence of eyPC (egg yolk PC) or bound to eyPC/bbPS (bovine brain PS) and eyPC/eyPG vesicles, the multiexponential data analysis results in five different lifetime components (see Table I), which remain constant as a function of wavelength. The fluorescence decay times shown in Table I are the average values for each of the five components obtained by averaging over the individual values at all examined wavelengths. On the contrary, the corresponding relative amplitudes of each component are wavelength-dependent as shown in Tables II and III. Despite this wavelength dependence of the relative amplitudes of the decay-times, no decay-associated emission spectra were calculated because the quite high lipid concentration (1 mM), which is necessary to bind more than 85% of all protein molecules to the membrane, causes a background signal which distorts the steady-state emission spectra. These spectra appear qualitatively identical for all investigated protein/lipid systems. The back-

TABLE I

Average fluorescence decay times of Trp41 of FX F1-86 bound to LUV made from natural lipids (egg yolk PC and PG (eyPC/eyPG 60:40), egg yolk PC and bovine brain PS (eyPC/bbPS 75:25)) or in the presence of non-binding PC-LUV. The average decay times are averages for each of the five components, obtained by averaging over the individual values at all examined wavelengths

Decay time, ns	PC	PC/PG	PC/PS
τ_1	0.16 ± 0.03	0.16 ± 0.05	0.17 ± 0.02
τ_2	0.64 ± 0.08	0.62 ± 0.10	0.61 ± 0.07
τ_3	2.67 ± 0.20	2.60 ± 0.20	2.57 ± 0.20
τ_4	5.46 ± 0.52	5.29 ± 0.15	5.46 ± 0.43
τ_5	23.1 ± 1.2	24.9 ± 1.1	24.3 ± 1.4

ground signal might be associated to the appearance of the long-lifetime component of about 25 ns.

Since no effect of different membrane compositions using natural lipids was observed on the fluorescence lifetimes of the FX F1-86 single tryptophan residue (Tables I–III), we tried to ascertain that no impurities of the natural lipids are responsible for the fact that we are not able to detect any lipid specific change in the fluorescence characteristics as it was detected for BF1². Thus, we repeated the time-resolved measurements with two sets of synthetic lipids, namely the dioleoyl and dilauroyl phospholipids. Either with the dilauroyl or dioleoyl set, data analysis reveals five components with nearly identical wavelength-independent decay times within the experimental error (see Table IV). The corresponding relative amplitudes vary with the wavelength (not shown). By comparing the results obtained for FX F1-86, either in the presence of the seven tested lipid systems or

TABLE II
Corresponding relative amplitudes of the decay times of FX F1-86 (see Table I) bound to eyPC/eyPG 60:40 vesicles (wavelengths in nm)

Amplitude, %	315	322.5	330	337.5	345	352.5	360	367.5	375	382.5	390	397.5	405
A ₁	20.4	16.3	14.0	11.8	10.6	10.5	10.5	10.6	8.5	8.2	12.0	6.5	8.2
A ₂	33.8	33.1	30.4	28.9	27.1	25.2	23.2	21.7	20.6	18.2	16.1	16.6	17.0
A ₃	31.2	27.4	26.3	29.2	31.2	34.7	36.3	37.1	39.2	38.3	43.1	38.5	47.7
A ₄	14.6	21.1	24.2	24.5	24.9	23.0	23.4	24.0	25.4	29.3	23.1	32.4	21.5
A ₅	–	2.0	5.1	5.6	6.2	6.6	6.6	6.6	6.3	5.7	5.7	6.0	5.6

TABLE III
Corresponding relative amplitudes of the decay times of FX F1-86 (see Table I) bound to eyPC/bbPS 75:25 vesicles (wavelengths in nm)

Amplitude, %	315	322.5	330	337.5	345	352.5	360	367.5	375	382.5	390	397.5	405
A ₁	16.3	15.2	14.5	10.5	20.5	11.8	11.6	10.7	7.4	9.7	9.3	7.5	8.2
A ₂	36.5	36.1	34.0	33.3	30.9	27.2	25.9	24.8	25.5	21.3	20.7	20.0	19.0
A ₃	17.9	24.4	25.8	25.0	31.1	31.9	34.1	33.1	33.7	34.7	41.4	38.7	37.1
A ₄	23.5	22.1	20.9	25.8	21.2	22.8	22.0	24.9	26.6	28.0	22.6	27.5	29.1
A ₅	5.8	2.2	4.8	5.4	6.0	6.3	6.4	6.5	6.8	6.3	6.0	6.3	6.6

complexed with calcium ions², no substantial difference in the fluorescence decay behaviour of FX F1-86 was detected. The only significant difference between the results obtained from FX F1-86 in the presence and absence of vesicles is the appearance of a long-lifetime component of about 25 ns. This component was detected for all investigated lipid systems, for the pure eyPC system (no protein binding) as well as for the six tested binding lipid systems (natural as well as synthetic lipids). In all the systems the decay time is nearly the same within the experimental error, and the pre-exponential amplitudes are comparable ($\approx 5\%$) for each detected wavelength in all systems. Moreover, we have recorded the emission intensity and its decay of a solution containing pure eyPC vesicles in the absence of proteins at an emission wavelength of 345 nm. The used lipid concentration (1 mM) was identical to those used in the experiments in the presence of protein. The determined total emission was about 7% compared to the fluorescence intensity in the corresponding experiment in the presence of FX F1-86. The lifetime analysis yielded four components with decay times of 0.1, 1, 6 and 30 ns. When analysing a set of measurements, the pre-exponential amplitudes of the 0.1 component appeared to be in a range of 80%. The contributions of each of the other individual components differed from experiment to experiment, but never exceeded 10%.

DISCUSSION

The catalytic activity of the prothrombinase complex depends on the lipid composition of the membrane, especially on the head group of the acid

TABLE IV

Average fluorescence decay times of Trp41 of FX F1-86 bound to LUV made from synthetic lipids (dilauroyl (DL) or dioleoyl (DO) phospholipids with choline, glycerol and serine head groups (dilauroyl-, dioleoylphosphatidylcholine (DLPC, DOPC); dilauroyl-, dioleoylphosphatidylglycerol (DLPG, DOPG); dilauroyl-, dioleoylphosphatidylserine (DLPS, DOPS))

Decay time, ns	DOPC/DOPG	DOPC/DOPS	DLPC/DLPG	DLPC/DLPS
τ_1	0.18 ± 0.01	0.17 ± 0.05	0.16 ± 0.03	0.15 ± 0.04
τ_2	0.49 ± 0.09	0.60 ± 0.07	0.55 ± 0.05	0.55 ± 0.07
τ_3	2.09 ± 0.72	2.59 ± 0.19	2.57 ± 0.25	2.62 ± 0.29
τ_4	5.31 ± 0.98	5.74 ± 0.56	5.48 ± 0.61	5.88 ± 0.57
τ_5	24.5 ± 3.2	28.5 ± 1.7	23.7 ± 2.0	24.1 ± 1.0

phospholipids. A possible explanation for this observation could be a specific lipid-induced change of the protein conformation. As pointed out above, there is experimental evidence that the head group of the anionic phospholipid influences the conformation of the N-terminal Gla domain of prothrombin in a lipid-specific manner. By comparing the fluorescence decay data obtained with BF1 complexed to calcium ions with BF1 bound to phospholipid vesicles of different compositions, it was shown that the most effective catalytic lipid phosphatidylserine has no influence on the fluorescence decay behaviour while with phosphatidylglycerol, a lipid with lower catalytic activity, an increase in the decay times assigned to the Gla tryptophan residue (Trp42) was observed.

To obtain detailed information about the mechanism of the prothrombinase reaction and its possible lipid specificity, the enzyme involved in the reaction should also be investigated. In the present work, fragment 1-86 of factor X bound to phospholipid vesicles of different compositions was studied by tryptophan time-resolved fluorescence spectroscopy. Data analysis of all investigated lipid systems revealed five decay components with nearly identical fluorescence lifetimes and relative amplitudes, within the experimental error. By comparing the results of the systems containing different anionic phospholipids with the eyPC reference system, no substantial change in the fluorescence lifetimes could be observed. The decay times are even comparable to those of FX F1-86 complexed with calcium ions (Ca-FX F1-86: 0.30, 0.65, 2.7 and 5.4 ns), but there was no long-lifetime component detected in the calcium experiment. In a recent publication we have discussed the origin of those four components found for Ca-FX F1-86 in detail². In brief, we have evidence that the components characterised by the fluorescence lifetimes of 0.65, 2.7 and 5.4 ns represent three different conformational states of the microenvironment of Trp41 in its uncomplexed (unquenched) form. The 0.30 ns component, on the other hand, might be assigned to electron transfer from the tryptophan-excited state to the disulfide bridge within the tryptophan–disulfide complex.

For all investigated lipid systems, a long-lifetime component of about 25 ns was found, but there were no significant differences in the pre-exponential amplitudes of this component, either for pure eyPC vesicles or for the binding systems. The appearance of such a long-lifetime component of about 25 ns is not usual in tryptophan fluorescence, although it has been shown that for some proteins a long lifetime appears when bound to lipid membranes¹³. Since there are no detectable differences in decay lifetimes or amplitudes between binding and non binding systems, the 25 ns component has to be explained rather by an artefact than by the influence of the bind-

ing of FX F1-86 to lipid membranes. The emission intensity of eyPC vesicles in the absence of proteins detected at 345 nm is about 7% compared to the fluorescence intensity in the corresponding experiment in the presence of FX F1-86. Since the decay time of the major components (80% pre-exponential amplitude) is about 0.1 ns and thus somehow shorter than the given time-resolution of the experiment (0.15 ns), a major part of this emission might be assigned to light scattering effects of the vesicle suspension. However, still a significant part of the fluorescence decay occurs on the nanosecond time scale and thus must be due to the fluorescence emission of the used phospholipid preparation. Let us note that this parasitic fluorescence appears when using natural as well as synthetic phospholipids.

The observation that all the lipid systems presently studied show comparable tryptophan fluorescence decay characteristics of FX F1-86 indicates that neither PG- nor PS-binding influences the microenvironment of the Gla tryptophan residue (Trp41) of FX F1-86. It could be possibly argued that it would be an overstatement if one rules out a PG induced conformational change by this observation. Considering the high sensitivity of the fluorescence of the Gla tryptophan residue to changes in the microenvironment as a consequence of a calcium-induced conformational change^{2,3} and the fact that we did find a PG-induced change in the fluorescence of the Gla tryptophan of prothrombin, we believe to have presently gathered reasons to speculate that PG alters the conformation of the Gla domain in prothrombin, but possibly not in factor Xa. Though this hypothesis should be supported by experiments using alternative techniques, we would like to point out that the structures of the Gla domains of BF1 and FX F1-86 differ regarding the influence of calcium binding on them. About apo-BF1 (BF1 without calcium), it is known that one third of its structure is disordered and that a more defined structure is obtained only after complexing the protein with calcium ions¹⁴. In this protein sequence the calcium-binding Gla domain follows a so-called "kringle" domain without high calcium affinity binding sites¹⁵. Binding of calcium ions to the Gla domain induces a closer approach of the Gla and kringle domains¹⁶ but the domain folding in the presence and absence of calcium ions remains nearly the same, with no essential differences¹⁴. It can be concluded that this intramolecular interaction of the Gla domain with the next situated kringle domain¹⁶ is only of minor importance. It could not be compared to the interaction between the Gla domain of fragment 1-86 of factor X and its C-terminal EGF-like domain, as will be described below. Generally, the Gla domain of the apo-FX F1-86 (FX F1-86 without calcium) shows a disordered structure and the Gla and EGF-like domains are flexible enough not to in-

teract with each other. High-affinity binding of calcium ions to the EGF-like domain locks the relative position of the Gla domain to the EGF-like domain^{17,18}. In the presence of calcium ions, the Gla and EGF-like domains show an interaction: The Gla domain induces a higher calcium affinity of the EGF-like domain, which in turn influences the properties of the Gla domain¹⁹. Such stabilisation and anchoring of the Gla domain by the C-terminal EGF-like domain is not found for BF1. Possibly, this stabilisation precludes a PG-induced change in the tryptophan fluorescence characteristics of the Gla domain of FX F1-86 as it is observed for BF1.

In conclusion, our findings show that the vesicle composition has no influence on the tryptophan fluorescence decay characteristics of FX F1-86. On the other hand, tryptophan fluorescence and FTIR studies indicate that the binding to PG-containing membranes alters³ the conformation of the Gla domain in prothrombin fragment 1. Concerning the catalytic activity of the prothrombinase, this result may suggest that additional experiments be performed in order to elucidate the role of the Gla domains of factor Xa and prothrombin, when searching for molecular reasons for the high lipid specificity observed in the prothrombinase reaction.

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REFERENCES

1. Jones M. E., Lentz B. R., Dombrose F. A., Sandberg H.: *Thromb. Res.* **1985**, 39, 711.
2. Häfner A., Merola F., Duportail G., Hutterer R., Schneider F. W., Hof M.: *Biopolymers* **2000**, 57, 226.
3. Hof M.: *Biochim. Biophys. Acta* **1998**, 1388, 143.
4. Wu J. R., Lentz B. R.: *Biophys. J.* **1991**, 60, 70.
5. Hof M., Fleming G. R., Fidler V.: *Proteins: Struct., Funct., Genet.* **1996**, 24, 485.
6. Persson E., Seelander M., Linse S., Drakenberg T., Öhlin A. K., Stenflo J.: *Biol. Chem. J.* **1989**, 264, 16897.
7. Esnouf M. P., Loyd P. H., Jesty J.: *Biochem. J.* **1973**, 131, 781.
8. Cutsforth G. A., Whitaker R. N., Hermans J., Lentz B. R.: *Biochemistry* **1989**, 28, 7453.
9. Brochon J. C., Tauc P., Merola F., Schoot B. M.: *Anal. Chem.* **1993**, 65, 1028.
10. Marquardt D. W.: *J. Soc. Ind. Appl. Math.* **1963**, 11, 431.
11. Hof M., Schleicher J., Schneider F. W.: *Ber. Bunsen-Ges. Phys. Chem.* **1989**, 93, 1377.
12. Livesey A. K., Brochon J. C.: *Biophys. J.* **1987**, 52, 693.
13. Döring K., Konermann L., Surrey T., Jähnig F.: *Eur. Biophys. J.* **1995**, 23, 423.
14. Soriano-Garcia M., Padmanabhan K., de Vos A. M., Tulinsky A.: *Biochemistry* **1992**, 31, 2554.

15. Lunblad R.: *Biochem. Biophys. Res. Commun.* **1988**, 157, 295.
16. Hamaguchi N., Charifson P., Darden T., Xiao L., Padmanabhan K., Tulinsky A., Hiskey R., Pedersen L.: *Biochemistry* **1992**, 31, 8840.
17. Sunnerhagen M., Olah G. A., Stenflo J., Forsen S., Drakenberg T., Trewhella J.: *Biochemistry* **1996**, 35, 11547.
18. Sunnerhagen M., Forsen S., Hoffren A. M., Drakenberg T., Teleman O., Stenflo J.: *Nat. Struct. Biol.* **1995**, 2, 504.
19. Stenflo J., Stenberg Y., Muranyi A.: *Biochim. Biophys. Acta* **2000**, 1477, 51.