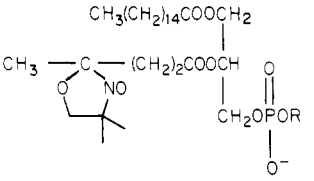
Asymmetric Lipid Fluidity in Human Erythrocyte Membrane: New Spin-Label Evidence†

Michel Seigneuret,‡ Alain Zachowski, Andreas Hermann,§ and Philippe F. Devaux*

ABSTRACT: We have synthesized spin-labeled analogues of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine with a short β chain (C5) bearing a doxyl group at the fourth position. When added to an erythrocyte suspension, the labels immediately incorporate in the membrane. The orientation of the spin-labels was assessed in the bilayer (i) by addition in the medium of a nonpermeant reducer (ascorbate at 5 °C) or (ii) by following spontaneous reduction at 37 °C due to the endogenous reducing agents present in the cytosol. Both techniques prove that the spin-labels are originally incorporated in the outer leaflet and redistribute differently after incubation. After a 5-h incubation at 5 °C, the phosphatidylcholine derivative remained in the outer layer, while the phosphatidylethanolamine and phosphatidylserine derivatives were found principally in the inner leaflet. During the incubation, a small fraction of the spin-labels is hydrolyzed, particularly the phosphatidylserine derivative, presumably by an endogenous phospholipase A_2. Because the hydrolyzed spin-labeled fatty acids are rejected in the aqueous phase, the spectra of the intact membrane-bound phospholipids can be obtained by an adequate spectral subtraction The ESR spectrum corresponding to a probe in the outer leaflet indicates a more restricted motion than that associated with probes in the inner leaflet. Additional experiments have been carried out to prove that the difference in viscosity, which is likely to be due to anisotropic cholesterol distribution, is not attributable to modification of the cell morphology. These results confirm the results on erythrocyte anisotropic fluidity suggested by Tanaka and Ohnishi [Tanaka, K. I., & Ohnishi, S. I. (1976) *Biochim. Biophys. Acta 426*, 218–231] and Williamson et al. [Williamson, P., Bateman, J., Kozarsky, K., & Mattocks, K. (1982) *Cell (Cambridge, Mass.) 30*, 725–733] but disagree with the results of Cogan and Schachter [Cogan, U., & Schachter, D. (1982) *Biochemistry 21*, 2146–2150].

In 1973, Bretscher showed that aminophospholipids are preferentially located on the inner surface of human erythrocyte membrane (Bretscher, 1973). Since then several lines of evidence have indicated an asymmetrical phospholipid distribution in various biological membranes [see the reviews by Op den Kamp (1979), Etemadi (1980), and Van Deenen (1981)]. This compositional asymmetry may be associated with differences in lipid mobility or "fluidity" between inner and outer leaflets. Such lipid motional asymmetry has indeed been reported in human erythrocytes (Tanaka & Ohnishi, 1976; Cogan et al., 1981; Williamson et al., 1982; Schachter et al., 1982) and in turkey erythrocytes (Rimon et al., 1980; Henis et al., 1982). However, some discrepancy appears in the literature. Tanaka & Ohnishi (1976) observed that the ESR spectrum of a spin-labeled phosphatidylcholine incorporated in intact erythrocytes was significantly broader than those obtained with the corresponding spin-labeled phosphatidylserine and phosphatidylethanolamine. Though the transbilayer distribution of the specific spin probes used for mobility measurements was not tested, the authors suggested a more rigid outer layer. This interpretation was supported by Williamson et al. (1982) by measurements of membrane binding of a fluorescent dye. However, fluorescence polarization studies by another group (Schachter et al., 1982; Cogan et al., 1981) have been interpreted as indicating greater lipid mobility in the outer leaflet. Similarly, fluorescence intensity measurements by Rimon et al. (1980) as well as photobleaching experiments by Henis et al. (1982), with turkey erythrocytes, seem to indicate a greater mobility of the outer leaflet. It is not obvious that these differences can be accounted for by differences in the spectroscopic techniques employed. On the other hand, it appears that the localization of the probes has not always been clearly established. For this reason we have carried out new experiments in human erythrocyte. The fluidity was investigated with spin-labeled phospholipids. The localization of the probe within the membrane was carefully controlled for each probe.

The following spin-labeled phospholipids have been used:



(0,2)PC, R = $(CH_2)_2\overset{+}{N}(CH_3)_3$

(0,2)PS, R = $CH_2\underset{\displaystyle COO^-}{\overset{\displaystyle \overset{+}{N}H_3}{CH}}$

(0,2)PE, R = $(CH_2)_2\overset{+}{N}H_3$

A significant advantage of such spin-labeled phospholipids is their partial water solubility, which allows an easy incorporation in biological membranes (Zachowski & Devaux, 1983). The nitroxide is not on the polar head group and thus should not perturb the head group specificity. On the other hand, because the free radical is not deeply buried within the membrane, selective chemical reduction can be used to assess the spin-labeled transbilayer distribution. Phospholipid exposure to the outer layer of erythrocytes can be demonstrated by addition of ascorbate to the suspending medium at low temperature (5 °C) (Kornberg & Mc Connell, 1971; Rousselet et al., 1976), while inner layer exposure is assessed by spon-

† From the Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, F-75005 Paris, France. *Received May 14, 1984.* This work was supported by grants from the Centre National de la Recherche Scientifique (ERA 690), the Ministère de l'Industrie et de la Recherche, the Université Paris VII, and the Fondation pour la Recherche Médicale.

‡ Present address: Centre d'Etudes Nucléaires de Saclay, Département de Biologie, Service de Biophysique, 91191 Gif-sur-Yvette Cedex, France.

§ Present address: Humboldt Universität zu Berlin, Sektion Biologie, Bereich Biophysik, 1040 Berlin, DDR.

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taneous reduction at high temperature (37 °C). The latter phenomenon is due to the reducing properties of the erythrocyte cytoplasmic content, i.e., very likely glutathione (Rousselet et al., 1976).

Materials and Methods

Erythrocytes, from fresh blood drawn on citrate–phosphate–dextrose, were washed 5 times with a 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, and 20 mM Hepes buffer, pH 7.4 (buffer A).

Cell hemolysis was performed by brief sonication (15 s) of the erythrocytes (75% hematocrit) at 0 °C using a 3 mm diameter titanium probe (Ultrasons, Annemasse).

Spin-Labeling. Phospholipid spin-labels were synthesized as already described (Devaux et al., 1975; Hubbell & Mc Connell, 1971; Seigneuret & Devaux, 1984). For labeling, packed erythrocytes were resuspended to a final hematocrit of 75% in buffer A to which the spin-label had been added from a concentrated ethanol solution. The suspension was then gently mixed. The spin-label concentration corresponded to approximately 1% of endogenous phospholipids.

Reduction Experiments at Low Temperature. Sodium ascorbate in buffer A was added to the sample at 5 °C, at a final concentration of 10 mM ascorbate, from a 10-fold concentrated stock solution adjusted to pH 7.4, followed by gentle mixing.

ESR spectra were recorded with a Varian E109 spectrometer equipped with a temperature control device and connected to a Tektronix 4051 computer. In order to record spectra of relatively dilute samples in a short period of time, a slight overmodulation (2 G) was systematically used. This resulted in a distortion of the very narrow components but had no consequence on the final conclusions, which are related to the membrane-bound components. Reduction of the ESR signal due to ascorbate or spontaneous reduction was monitored generally by following a specific peak intensity (e.g., the medium field line). Alternatively, the total spectrum was recorded as a function of time and integrated to obtain the spin-label concentration. The two procedures gave very close results, indicating that as a first approximation ESR shape changes (see Results) can be neglected when studying the reduction kinetics.

The morphology of the erythrocytes after incubation with spin-labels was monitored under a light microscope. Labeled samples were fixed by dilution to a 5% hematocrit in an ice-cold 2% glutaraldehyde solution in sample buffer, incubated on ice for 1 h, and viewed under a Zeiss WL phase contrast microscope with a total magnification of 800.

Results

ESR Spectra of Spin-Labeled Phospholipids in Erythrocyte Membranes. The ESR spectrum of 0.125 mM (0,2)PC in buffer A at 5 °C is shown in Figure 1. Identical spectra were obtained for (0,2)PE and (0,2)PS. The spectrum is composed of a broad component corresponding to phospholipids in micelles and a minor narrow component that we attribute to monomers. If the spectrum is recorded with lower spin-label concentration, the ratio of the broad vs. narrow component decreases, confirming the above hypothesis (critical micellar concentration $\simeq$ 10 μM). In Figure 1b, the ESR spectrum of (0,2)PC and (0,2)PS is shown *3 min after* the addition of the spin-labels to an erythrocyte suspension at 5 °C. The spectrum has a completely different line shape. The absence of spin–spin interactions reveals the immediate incorporation of both labels in the membranes. Note that the narrow component has completely disappeared. Under identical conditions,

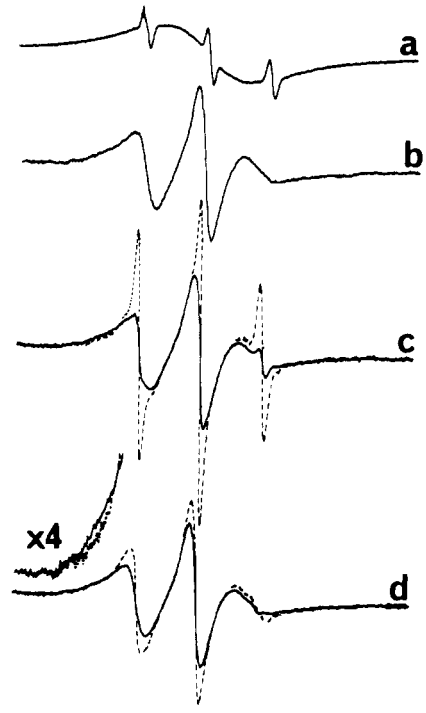

FIGURE 1: ERS spectra of (0,2) phospholipids (5 °C): (a) (0,2)PC in buffer A; (b) 3 min after addition to erythrocytes [identical spectra are obtained with (0,2)PS and (0,2)PE]; (c) 4 h after addition to erythrocytes [solid line, (0,2)PC; dotted line, (0,2)PS]; (d) same as in (c) but after computer subtraction of the narrow component.

(0,2)PE gives the same result.

Figure 1c shows the results of a 4-h incubation at 5 °C of (0,2)PC and (0,2)PS in erythrocyte membranes. Both spectra again exhibit a narrow component, which is larger for (0,2)PS (dotted line) than for (0,2)PC (solid line). A similar observation was made when the labels were incorporated in erythrocyte ghosts. This narrow component is *not* due to the spin-labeled phospholipids being resolubilized in water upon incubation. Rather it is due to *hydrolysis* of a fraction of the spin-labeled phospholipids. Indeed it has been shown that human erythrocytes contain an endogenous phospholipase A, which is activated by sublytic amounts of detergent (Paysant et al., 1970). Moreover, when 4-h-incubated erythrocytes were centrifuged, the supernatant gave rise only to a narrow ESR spectrum. When this supernatant was added back to unlabeled cells, the ESR spectrum remained narrow, clearly indicating that the spin-label did not incorporate into the membrane as a (0,2) phospholipid would. That the narrow component is due to free fatty acid formation can be further proved by computer subtraction of the spectrum obtained (under the same modulation conditions) with the corresponding 5-carbon spin-labeled fatty acid in water.[1] Double integration of the narrow component present in Figure 1c allows a quantitative estimation of the percentage of hydrolysis: hydrolysis is less rapid for (0,2)PC (1.7% in 4 h) than for (0,2)PS (11% in 4 h) and (0,2)PE (4.3% in 4 h).[2]

Subtracting the contribution of the free fatty acids yields the two spectra superimposed in Figure 1d. The spectrum associated with (0,2)PE is similar to that of (0,2)PS (dotted line). The two spectra are normalized to the same double

[1] Best matching of the narrow component is obtained with free fatty acid solubilized in erythrocyte hemolysate.

[2] Note that the same type of subtraction did not allow us to erase completely the narrow component in Figure 1a, thereby confirming that the narrow component in Figure 1a is not due to hydrolyzed fatty acid but rather to monomeric spin-labeled phospholipids tumbling in water.

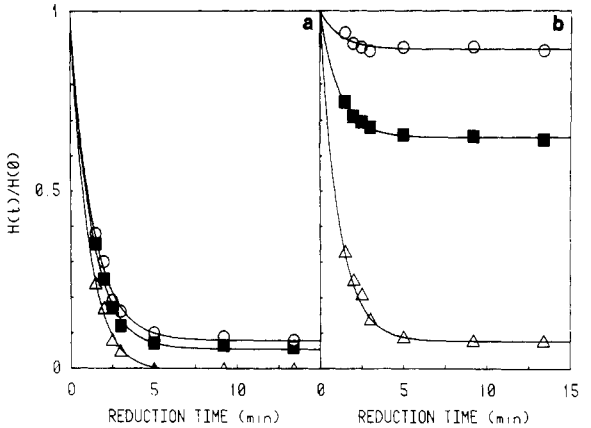

FIGURE 2: Reduction kinetics by ascorbate of the ESR signal arising from various (0,2) phospholipids incorporated into erythrocyte membranes. The amount of signal was expressed as the ratio of the central line height at a given time to the height before addition of ascorbate. In (a) ascorbate was added after a 3-min incubation at 5 °C. In (b) incubation lasted 4 h at 5 °C before ascorbate addition. (△) (0,2)PC; (■) (0,2)PE; (○) (0,2)PS.

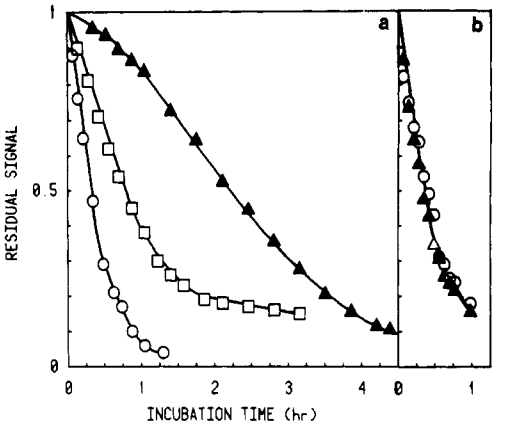

FIGURE 3: Spontaneous reduction at 37 °C of the signal arising from (0,2) phospholipids incorporated into erythrocyte membranes. The spin-label was added to the cell prewarmed at 37 °C, and then the ESR spectrum was sequentially recorded. In (a) the experiment was performed with intact cells. In (b) cells lysed by sonication were used. (▲) (0,2)PC; (□) (0,2)PE; (○) (0,2)PS.

integral. It can be seen that a significant difference in line shape between (0,2)PC and (0,2)PS [or (0,2)PE] has appeared upon incubation. While the spectrum of the membrane-bound (0,2)PC is unchanged relative to that initially observed, the spectrum of (0,2)PS [or (0,2)PE] indicates more motion.

Transbilayer Distribution of Spin-Labeled Phospholipids: Its Evolution with Time. Several experiments were carried out to assess the localization of the various spin-labels in the erythrocyte membrane. Figure 2 shows the result of reduction by ascorbate. Figure 2a shows the kinetics of reduction of (0,2)PC, (0,2)PS, and (0,2)PE, as obtained shortly after addition of the labels to the membrane. For the three phospholipids, the plateau is very near zero, revealing that the probes are initially incorporated in the outer layer. Thus the spectrum in Figure 1b corresponds to the viscosity of the *outer layer*. Because the line shape in Figure 1b is independent of the head group, one can conclude that the three spin-labels explore the same lateral domain. After 4-h incubations at 5 °C (Figure 2b) the distribution of the spin-labels is quite different: 90% of (0,2)PS and 75% of (0,2)PE are now unaccessible to ascorbate, while only about 10% of (0,2)PC is protected. This spontaneous and selective redistribution of labeled phospholipids allows us to conclude that the spectrum of (0,2)PS in Figure 1d (i.e., after a few hours' incubation) is that of a spin-label in the inner layer of erythrocyte membranes while the spectrum of (0,2)PC is due to a spin-label in the outer layer. Hence the fluidity appears to be greater in the inner layer.

To further assess the redistribution of the spin-labeled phospholipids, we have recorded the spontaneous reduction that takes place at 37 °C. The difference between the reduction rates of the three spin-labeled phospholipids is striking (Figure 3a). Results in Figure 3b are obtained with cells lysed by sonication immediately after labeling. This time the three spin-labels are reduced practically at the same rate. The half reduction time is very close to that obtained with intact cells for (0,2)PS. Thus the rate-limiting step for (0,2)PS reduction in intact cells is not the diffusion of PS toward the inner layer but rather the chemical reduction by the cell interior. It must be stressed that no such reduction with either intact cells or lysed cells takes place at 5 °C. In conclusion, the kinetics of spontaneous reduction at 37 °C suggest that the PS derivative is very rapidly transported toward the inner layer (half-time <15 min). PE is transported at a slightly slower rate, while PC flips with a half-time of 2 h at 37 °C.[3] These results confirm the conclusions reached concerning the asymmetrical redistribution of spin-labeled phospholipids from reduction experiments carried out at 5 °C.

Is the Change in ESR Line Shape Related to a Change of Cell Morphology? Fresh erythrocytes before incubation with spin-labels had a normal discocyte shape. However, immediately after addition of the spin-labeled phospholipids, all cells turned into echinocyte shape. The echinocyte shape is stable for several hours if (0,2)PC is used. On the other hand, a progressive change into discocyte and stomatocyte is observed upon incubation with either (0,2)PC or (0,2)PS. These findings are entirely consistent with the "bilayer couple hypothesis" of Sheetz & Singer (1977) and allowed us (in a different work) to use the variations of cell morphology to measure the flip-flop rates of the spin-labels (Seigneuret & Devaux, 1984). Thus, the spectrum displayed in Figure 1b is associated with probes in the outer layer and simultaneously with cells in the echinocyte shape; the dotted curve in Figure 1d is associated with (0,2)PS [or (0,2)PE] mainly in the inner layer and cells in the stomatocyte shape. One may ask if the fluidity changes, which we have attributed in the above lines to the intrinsic difference in fluidity of both layers, are in fact caused by the difference in constraint on the whole membrane associated with different shapes.

The following experiments were then carried out: (i) (0,2)PS was added to fresh erythrocytes at 5 °C at a final spin-label to phospholipid ratio of 1%. Cells were allowed to incubate 2 h at 5 °C; then ascorbate was added. At that stage cells were partially in the echinocyte shape and partially in the discocyte or stomatocyte shape. Nevertheless, the final ESR spectrum, *which must correspond to the spin-labels in the inner layer*, was identical with the dotted spectrum of Figure 1d. This is the first indication that the ESR line shape is independent of cell shape. (ii) To the same cells that had been labeled with (0,2)PS was added a high concentration of (0,2)PC (final spin-label to phospholipid ratio 6%). All cells turned into echinocytes. Ascorbate was added, resulting in chemical reduction of the (0,2)PC and of the (0,2)PS located in the outer leaflet but leaving the cells in the echinocyte shape.

[3] It cannot be ruled out that part of the phosphatidylcholine reduction, which takes place after several hours' incubation at 37 °C, is due to lysed cells. Also hydrolysis is activated at this high temperature; the free fatty acid can penetrate easily in the cells and be reduced.

Yet the final spectrum was identical with the dotted spectrum of Figure 1d. Thus the spectra associated respectively with the outer or inner layers are independent of the cell shape.

Discussion

The ascorbate experiments, as well as the kinetics of spontaneous reduction by the cytosol, clearly indicate that all three spin-labeled phospholipids used in this investigation initially incorporate in the outer leaflet of the membrane. This behavior is consistent with that of fluorescent phospholipids also bearing a short labeled chain (Pagano et al., 1981). No exchange protein is required to shuttle phospholipid between the micelles and the membrane. Very likely the easy incorporation is due to the presence of a fraction of monomers in solution. By contrast, long-chain spin-labeled phospholipids are incorporated in erythrocytes, with a low efficiency, by a vesicle fusion process that distributes the labels equally between both leaflets (Rousselet et al., 1976).

Upon incubation, the spin-labeled phospholipids are shown to spontaneously adopt a transbilayer distribution that is similar to that of the corresponding endogenous phospholipids of the erythrocyte membrane. The molecular mechanisms responsible for the formation and maintenance of lipid asymmetry in erythrocyte membranes are the subject of different hypotheses (Heast et al., 1978; Seigneuret & Devaux, 1984). In the present paper we emphasize the results concerning the membrane fluidity rather than the mechanism by which the probes are directed toward their specific target. Consequently, we limit the discussion to the interpretation of the ESR line shapes associated respectively with the outer and inner leaflets. The spectra in Figure 1d show a significant difference, which can be interpreted as revealing more motion in the inner layer. Unfortunately, both spectra are very difficult to analyze at a quantitative level. Such spectra correspond to motions in the so-called "intermediate regime" where order parameters and rates of motions are influencing the line shape. In addition, it is impossible to prove that the spectra are homogeneous, i.e., correspond to a single environment. Finally, the probe is at a single position of a short acyl chain and therefore does not report completely on the mobility of each leaflet. Having in mind these fundamental difficulties, one can nevertheless attempt to express the physical differences between both layers in a semiquantitative form. A first approximation can be obtained by the formula of Keith et al. (1970), valid for fast isotropic motions. By such formula we obtain an effective correlation time of 3.61 ± 0.09 ns for all three labels in the outer layer at 5 °C. After diffusion of (0,2)PS and (0,2)PE toward the inner layer, a value of 2.69 ± 0.08 ns is obtained for the two labels. This corresponds to a 25% decrease of the apparent correlation time. Alternatively one can say that the "effective temperature" of the outer layer is about 10 °C below that of the inner layer. Indeed, to obtain the dotted curve in Figure 1d with the PC derivative necessitates a 10 °C increase of temperature. In conclusion, we are well aware that the general concept of membrane fluidity is lacking a rigorous definition. Nevertheless, it is an accepted term used to express the relative ease with which a free molecule tumbles in the hydrophobic core of a membrane. With this definition in mind, we conclude that the fluidity of the inner layer in erythrocytes is larger than that of the outer layer. These results confirm unambiguously the results of Tanaka & Ohnishi (1976) obtained with long-chain spin-labels.

What is new in our contribution? In order to probe the viscosity of the membrane, Tanaka & Ohnishi (1976) have used spin-labeled phospholipids with a nitroxide at the 12th carbon position of a stearoyl chain. As a result, the probe could not be reached by ascorbate and they were unable to verify the distribution of their probe in the erythrocyte membrane. On the other hand, they have attempted to assess the distribution of phospholipids spin-labeled on the head group. With Fe–cysteine the reduction was shown to be very rapid for the phosphatidylcholine analogue and slow for the phosphatidylserine analogue. However, both analogues bore distinct nitroxide moieties. The former was a six-membered ring and the latter a five-membered oxyoxazolidine ring. We have shown previously (Rousselet et al., 1976) that the phosphatidylcholine analogue used by Tanaka and Ohnishi is immediately reduced even at 0 °C when in contact with the erythrocyte cytosol, so that further reduction must be entirely due to the labels outwardly exposed. On the other hand, the phosphatidylserine analogue used by Tanaka and Ohnishi is likely to resist reduction from the cytosol since, as pointed out by the authors, it is only reduced by Fe–cysteine and not by ascorbate. Thus the difference in reactivity of the two labels can entirely account for the difference in reduction kinetics reported by these authors. In conclusion, their work cannot be used as a demonstration of the difference in fluidity of the inner and outer leaflet of the erythrocyte membrane. The present work does not suffer from this criticism since the same class of nitroxides is borne by the three phospholipids that are used to measure viscosity as well as probe orientation within the membrane.

Our results are in agreement with the work of Williamson et al. (1982) using a dye binding method but not with the fluorescence polarization data of Schachter and collaborators (Cogan & Schachter, 1981; Schachter et al., 1982). While we have no precise explanation for such discrepancy, it may be remarked that other authors have stressed that fluorescence polarization data on erythrocytes and ghosts may be hampered by artifacts due to light scattering as well as to the ghosting procedure (Aloni et al., 1974; Kutchai et al., 1982). Finally, our results are at variance with those of Rimon et al. (1980) and Henis et al. (1982). However, the latter results concern turkey and not human erythrocytes.

What is the reason for the increased viscosity of the outer membrane? Cholesterol, whose rigidifying effect in liquid-crystalline bilayers is well-known, appears to be distributed mainly in the outer layer (Fisher, 1976; Hale & Schroeder, 1982). This would explain the spin-label data. However, there are some disagreements again in the literature concerning this point (Blau & Bittman, 1978). An alternate explanation would be that the difference of viscosity is associated with the difference in unsaturation of phosphatidylcholine compared to phosphatidylserine and phosphatidylethanolamine in human erythrocytes (Williams et al., 1966). Although in LM fibroblasts the more rigid layer (the inner layer) is enriched in unsaturated fatty acids (Fontaine & Schroeder, 1979).

Registry No. (0,2)PC, 85284-79-3; (0,2)PS, 91280-60-3; (0,2)PE, 91266-01-2.

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Amino Acid Sequence of Phosvitin Derived from the Nucleotide Sequence of Part of the Chicken Vitellogenin Gene†

B. Marion Byrne,* Alfred D. van het Schip, Jos A. M. van de Klundert,‡ Annika C. Arnberg, Max Gruber, and Geert AB

ABSTRACT: The amino acid sequence of the egg yolk storage protein phosvitin has been deduced from the nucleotide sequence of part of the chicken vitellogenin gene. Of the phosvitin sequence, 210 amino acids including the N-terminal residue are contained on one large exon, whereas the remaining six amino acids are encoded on the next exon. Phosvitin contains a core region of 99 amino acids, consisting of 80 serines, grouped in runs of maximally 14 residues interspersed by arginines, lysines, and asparagines. The serines of the core region are encoded by AGC and AGT codons exclusively and the arginines by AGA and AGG, which results in a continuous stretch of 99 codons with adenine in the first position. The N-terminal quarter of the phosvitin sequence contains 16 serines grouped in a cluster with alanines and threonines and coded mainly by TCX triplets. The C-terminal part includes 27 serines, preferentially coded by AGC and AGT, 13 histidine residues, and the sequence ...Asn-Gly-Ser... at which the carbohydrate moiety of phosvitin is attached. Heteroduplex formation between cloned DNAs from chicken and *Xenopus* vitellogenin genes shows that the phosvitin sequence contains a stretch of highly conserved sequence.

Phosvitin is an egg yolk protein derived from a large precursor molecule, vitellogenin, which is formed in the liver of oviparous vertebrates under estrogen induction. Containing 10% phosphorus, it is one of the most highly phosphorylated proteins in nature (Levene & Alsberg, 1901; Mecham & Olcott, 1949). The phosphate is monoesterified to mainly serine residues (Lipmann & Levene, 1932; Lipmann, 1983) by an as yet unidentified kinase. Serine accounts for more than 55% of the amino acids in this phosphoprotein (Allerton & Perlmann, 1965). Evidence for the grouping of serines in runs, sometimes of six or more residues long, was found (Williams & Sanger, 1959).

Clark (1970) fractionated hen phosvitin into two discrete phosphoprotein species with apparent molecular weights of 3.4×10^4 and 2.8×10^4. The amino acid sequence of the larger, more abundant, species has been partially determined at the N-terminus (Clark, 1973) and at the site of glycosylation (Shainkin & Perlmann, 1971). A highly phosphorylated fragment of composition $Ser_{43}Asp_2Lys_3Arg_3$ has been isolated by Posternak & Waegell (1964). Determination of the complete amino acid sequence has proven difficult because of high resistance to proteolytic cleavage and the unusual amino acid composition of phosvitin (Posternak & Waegell, 1964; Belitz, 1965; Clark, 1973). Clark & Dijkstra (1980) have recently constructed derivatives of phosvitin to facilitate sequencing.

For our studies on the mechanism of steroid-controlled gene expression, we isolated clones covering the 21-kb[1] vitellogenin gene from a chicken DNA library and established its exon–intron organization (Arnberg et al., 1981). Comparison with

† From the Department of Biochemistry, Groningen University, Nijenborgh 16, 9747 AG Groningen, The Netherlands. *Received July 3, 1984.* This work was carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

‡ Present address: Laboratory for Medical Microbiology, Department of Bacteriology, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands.

[1] Abbreviations: kb, kilobase(s); bp, base pair(s); kDa, kilodalton(s); CNBr, cyanogen bromide.

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