Interactions between retinyl phosphate and bivalent cations

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In the presence of Mn(II) ions, the u.v. absorption spectrum of retinyl phosphate (Ret-*P*) solubilized in Triton X-100 micelles, phosphatidylcholine liposomes or rat liver microsomes exhibited a shift from the maximum of 330 nm to 287 nm. The effect of Mn(II) was reversed by adding EDTA or phosphate buffer. The same spectral change was found in the presence of poly-L-lysine in place of Mn(II) ions. The e.s.r. spectrum of Mn(II) in the presence or in the absence of Ret-*P* clearly showed that approx. 75% of the initial concentration of Mn(II) ions is bound to Ret-*P* when the molar ratio of Ret-*P* to Mn(II) ions is 4:1; no such binding occurred in the presence of retinol or retinoic acid. The appearance of two isosbestic points at 303 and 368 nm, in the presence of Mn(II) ions, suggests the existence of an equilibrium between an Mn(II)-bound monomer and an Mn(II)-bound dimer of Ret-*P* in Triton X-100 micelles. The same effect on the u.v.-absorption spectrum of Ret-*P* was also induced by Co(II), Cr(II), Zn(II) and Fe(II), but not by Mg²⁺ or Cu(II). The formation of the 'metachromatic complex' between Ret-*P* and Mn(II) or Co(II) inhibited the synthesis of retinyl phosphate mannose (Ret-*P*-Man) from exogenous and endogenous Ret-*P* and guanosine diphosphate [¹⁴C]mannose when bovine serum albumin was added after the metal ion. However, the order of addition did not influence Ret-*P*-Man synthesis in incubations containing MgCl₂, which does not form the metachromatic complex with Ret-*P*. These results suggest that the bioavailability of proteins, polyamines and metal ions may control the extent to which Ret-*P* can be mannosylated in the intact membrane.

Vitamin A has a unique chromophore of five conjugated double bonds that play an important role in the visual cycle (Wald, 1968; Morton, 1972). Recently, the phosphorylated vitamin has been shown to be involved in mannosyl-transfer reactions in microsomes (De Luca, 1977), suggesting a second mechanism by which vitamin A may function in the

body. Exogenous (Shidoji & De Luca, 1981) and endogenous (De Luca *et al.*, 1982) Ret-*P* accept mannose from GDP-mannose to form Ret-*P*-Man, which donates mannose directly to endogenous protein acceptors in the microsomal membrane (Creek *et al.*, 1983). Manganese ions stimulate the first mannosyl transfer reaction in which Ret-*P* functions as a substrate. Therefore it was of interest to look at the interaction between Ret-*P* and Mn(II) ions in hydrophobic environments.

Materials and methods

Ret-*P* was obtained by chemical phosphorylation of all-*trans*-retinol (Hoffmann–LaRoche, Nutley, NJ, U.S.A.) (Bhat *et al.*, 1980). Retinoic acid was a gift from Hoffmann–LaRoche Inc. DPPC was purchased from Cal-Biochem. Poly-L-lysine was purchased from Chemical Dynamics Corporation, South Plainfield, NJ, U.S.A. Other chemicals used were all reagent grade.

Abbreviations used: Ret-*P*, retinyl phosphate; Ret-*P*-Man, retinyl phosphate mannose; DPPC, (dipalmitoyl)phosphatidylcholine (dipalmitoyl)glycerophosphocholine); BSA, bovine serum albumin; medium A, 50 mM-Tris/HCl (pH 7.6), containing 5 mM-MgCl₂, 25 mM-KCl and 0.25 M-sucrose.

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U.v.-absorption spectra of Ret-*P* were recorded with a Beckman u.v. spectrophotometer (model 35) at room temperature. The molar absorption coefficient of Ret-*P* (ammonium salt) was 52 700 in methanol or in 0.1% Triton X-100, 33 400 in 50% methanol and 25 470 M⁻¹·cm⁻¹ in water, all at 325 nm. The concentration of Ret-*P* was measured spectrophotometrically at 325 nm in methanol ($\epsilon_{1\text{cm}}^{1\%}=1440$). E.s.r. spectra were recorded with a Varian model 102 X-band spectrometer with 100 kHz field modulation at room temperature (23–25°C). Phosphatidylcholine liposomes were prepared by published procedures (Hanover & Lennarz, 1978). Synthetic DPPC (2 mg/ml methanolic solution; 0.37 ml) was transferred to a test tube and evaporated to dryness under an N₂ stream. The residue was redissolved in 80 μ l of ethanol. The ethanolic solution was dispersed in 1 ml of 30 mM-Tris/HCl buffer (pH 8), and was incubated at 37°C for 1 h to make stable liposomes. Ret-*P* was solubilized in the liposomal suspension.

Preparation of rat liver microsomal vesicles and Ret-P-Man synthesis

Normal male rats, Osborne–Mendel strain (weighing 150–200 g), starved overnight, were killed by bleeding under a light diethyl ether anaesthesia. The livers were homogenized in 2 vol. of medium A with a glass/Teflon homogenizer. After centrifugation at 2500 *g* for 20 min, the resultant supernatant was centrifuged at 105 000 *g* for 60 min. The pellet was resuspended in 0.9% NaCl. Standard incubations were prepared as follows. GDP-[¹⁴C]-mannose (0.1 μ Ci) and 10 μ g of Ret-*P* in 99% methanol were transferred to test tubes. After removing the solvent under an N₂ stream, 4 mg of BSA/ml and the following compounds in that sequence were added in a final volume of 200 μ l in the indicated final concentration: 22.5 μ M-GDP-D-mannose; 30 mM-Tris/HCl buffer (pH 8); 5 mM-bivalent cation; 8 mM-NaF. 2 mM-ATP, 5 mM-AMP; 1 mg of the microsomal protein. Since BSA binds Ret-*P* so as not to permit the formation of the 'metachromatic' Ret-*P*–metal complex, it was added as the last component in incubations under column IV of Table 1. For incubations containing 0.1% Triton X-100, the detergent was added immediately after addition of BSA. Other ingredients were added in the same order as stated above. The mixture was incubated at 37°C for 2 min. The reaction was stopped by addition of 5 vol. of chloroform/methanol (2:1, v/v). The lower phase was dried, immediately redissolved in methanol containing 10–20 μ g of Ret-*P* and applied to thin layers of silica gels, which were chromatographed in chloroform/methanol/water (45:35:6, by vol.). The silica gel was scraped and radioactivity measured in Econofluor from New England Nuclear.

Results

Effects of Mn(II) on absorption spectra of Ret-P

Fig. 1 shows the absorption-spectral changes of Ret-*P* caused by an increase in the molar ratio (from 1 to 22.7) of Mn(II) ions to Ret-*P* in the presence of 0.1% Triton X-100 as a solubilizing agent. In the absence of Mn(II) ions, most Ret-*P* molecules are incorporated and dispersed randomly in Triton X-100 micelles without interaction between the chromophores, as suggested by the fact that the absorption spectrum of Ret-*P* in Triton X-100 micelles was quite similar (5 nm red shift) to that in methyl alcohol. However, in the presence of Mn(II) ions, the u.v. absorption spectrum of Ret-*P* exhibited spectral changes (Fig. 1) characteristic of the so-called 'metachromasy' (Yamaoka & Takatsuki, 1978). Therefore the term 'metachromasy' will be used in a broader sense to indicate such spectral changes of Ret-*P* in the presence of bivalent cations. No spectral changes of retinol or retinoic acid were found in the same conditions.

As shown in Fig. 2, the u.v.-absorption maximum at 330 nm could be restored by adding excess EDTA

Fig. 1. Effects of Mn(II) ion on absorption spectra of Ret-*P* (22 μ M) in 0.1% Triton X-100 micellar solution containing 10 mM-Tris/HCl (pH 8)

The concentration of Mn(II) ions are (A) 40 μ M, (B) 25 μ M (C) 50 μ M and (D) 500 μ M.

Fig. 2. Reversibility of the Mn(II)-induced spectral change of Ret-P

(1) 6.5 μ g of Ret-P was solubilized in 1 ml of 0.1% Triton X-100/10 mM-Tris/HCl (pH 8). Then, (2) 10 μ l of 0.1 M-MnCl₂, (3) 20 μ l of 0.1 M-Na₂EDTA and (4) 30 μ l of 0.1 M-MnCl₂ were sequentially added.

to the Ret-P/Mn(II) mixture. Moreover, re-addition of excess Mn(II) induced the same spectral changes (Fig. 2), suggesting that no chemical modification such as oxidation or hydrolysis had occurred on the

Fig. 3. Effect of poly-L-lysine on the absorption spectrum of Ret-P

(A) A methanolic solution (40 μ l) of Ret-P (6.5 μ g) was dispersed in 1 ml of 1 mM-Tris/HCl (pH 8); (B), 100 μ g of L-lysine was added to (A); (C), the same as (A); (D), 100 μ g of poly-L-lysine was added to (C).

Ret-P molecule in the presence of Mn(II) ions. A similar result was obtained by using 20 mM-sodium phosphate buffer, pH 7.0, instead of EDTA (not shown), indicating that the phosphate is involved in Mn(II)-induced spectral change of Ret-P.

Since 'metachromasy' is usually observed when staining dyes bind to polyelectrolytes or biopolymers (Yamaoka & Takatsuki, 1978; Takatsuki, 1980), we added a polycationic polymer, poly-L-lysine, in place of Mn(II) ions to a solution of Ret-P. A similar spectral change of Ret-P was exhibited as in the presence of Mn(II) ions (Fig. 3), indicating that Mn(II) ions play a similar role to the charged amino groups of polylysine to induce the appearance of 'metachromasy' of Ret-P. The perturbation of the conjugated double-bond system was apparently caused by the interaction between neighbouring chromophores themselves on poly-L-lysine, rather than by an interaction between Ret-P and the charged amino group. In fact our preparation of Ret-P contains ammonium as the counterion and free lysine did not give any change of the u.v.-absorption spectrum of Ret-P (Fig. 3).

In order to clarify whether or not Mn(II) ions bind to Ret-P molecules, e.s.r. spectra of Mn(II) were taken in the absence or in the presence of Ret-P. As shown in Fig. 4, a decrease in the intensity of the e.s.r. spectrum of Mn(II) was observed in the

presence of Ret-*P* in the Triton X-100 system. Since it is known that the e.s.r. spectrum of Mn(II) ions bound to functional groups becomes so broad that the spectrum is almost unobservable (Hyde *et al.*, 1979), the decrease in the intensity by three-quarters, compared with the spectrum obtained in the absence of Ret-*P*, suggests that approx. 75% of the initial concentration of Mn(II) ions bind to Ret-*P* molecules when the molar ratio of Ret-*P* to Mn(II) ions is 4:1. It is noteworthy that the same concentration of retinoic acid did not affect the e.s.r. spectrum of Mn(II).

Mn(II)-induced 'metachromasy' of Ret-P in phosphatidylcholine liposomes and rat liver microsomes

It was of interest to investigate whether or not Mn(II) ions can induce 'metachromasy' of Ret-*P* in phosphatidylcholine liposomes, because when Ret-*P* molecules are incorporated into liposomes, they are surrounded by the excess phosphate groups of

phosphatidylcholines. When Ret-*P* (27 μ M) is solubilized into a DDPC (1 mM) liposome suspension, Ret-*P* molecules are actually incorporated in the liposomes, as confirmed by the fact that Ret-*P* in the liposome suspension is eluted at the void volume of a Sepharose 4B column (results not shown). The appearance of 'metachromasy' in the presence of Mn(II) ions (Fig. 5*a*) clearly indicates that Mn(II) ions can interact with Ret-*P* even in the phosphatidylcholine liposomes.

This result implies that the Mn(II) ion can recognize the phosphate groups of Ret-*P* even among the polar head-groups of phosphatidylcholines. Since the main difference in the charged groups between phosphatidylcholine and Ret-*P* is that the groups of phosphatidylcholine are amphoteric, whereas the group of Ret-*P* is anionic, it may be that the Mn(II) complex with Ret-*P* is more stable electrostatically than that with phosphatidylcholine.

The same experiment as above was performed with the use of rat liver microsomes instead of phosphatidylcholine liposomes. The results are shown in Fig. 5(*b*), where 'metachromasy', as shown in Figs. 1 and 5(*a*), was observed in the presence of Mn(II) ions. Thus, even in biological membranes, Mn(II) ions can induce the observed spectral changes.

The micellar structure or lipid bilayer seems to be necessary for the spectral change of Ret-*P*, probably for a parallel stacking of the conjugated double-bond systems. In fact, when Ret-*P* was solubilized in BSA solution, we failed to observe the spectral change of Ret-*P* after addition of Mn(II) ions (Fig. 6). Since we utilize the BSA system for synthesis of Ret-*P*-Man, this finding would suggest that the 'metachromatic complex' of Ret-*P* and Mn(II) is not necessary for the synthetic activity.

Fig. 4. E.s.r. spectra of Mn(II) ions in the absence (*a*) and in the presence (*b*) of Ret-*P* in 4.5% Triton X-100 micellar solution

The initial concentration of Mn(II) ions is 0.25 mM.
The molar ratio of Ret-*P* to Mn(II) ions is 4:1.

Table 1. Formation of a complex between Ret-*P* and metal ions inhibits Ret-*P*-Man synthesis

Incubations contained 0.1 μ Ci of GDP-[14 C]mannose (22.5 μ M) and 10 μ g of Ret-*P* in addition to other components specified in the Materials and methods section. Incubations were conducted in the presence of either 2.5 mM or 5 mM metal ion. In column IV the metachromatic complex was allowed to form before addition of BSA.

14 C radioactivity in Ret-*P*-Man (c.p.m./mg of protein per 2 min)
from lower phase of:

	5 mM		2.5 mM		Ret- <i>P</i> 'metachromasy' in Triton X-100 micelles
	I Ret- <i>P</i> /BSA	II Triton assay	III Ret- <i>P</i> /BSA	IV Ret- <i>P</i> /metal	
CoCl ₂	10200	7440	10500	2930	+
MgCl ₂	11040	9330	4670	4040	—
MnCl ₂	4750	2050	11600	2800	+
ZnCl ₂	580	280	—	—	+
FeCl ₂	630	960	—	—	+
CuCl ₂	530	500	—	—	—
CrCl ₂	520	1101	—	—	+
CaCl ₂	960	480	—	—	+

Fig. 5. Effect of $Mn(II)$ ions (1 mM) on the absorption spectrum of (a) Ret-P (27 μM) in 1 mM-DPPC liposome suspension containing 10 mM-Tris/HCl (pH 8) at room temperature and (b) effect of $Mn(II)$ ions (0.5 mM) on the

Effect of other bivalent metal ions on absorption spectra of Ret-P

In the presence of Ca^{2+} , $Zn(II)$, $Fe(II)$, $Cr(II)$ or $Co(II)$ ions, the same 'metachromasy' as shown in Fig. 1 was observed in Triton X-100 micellar solution. In sharp contrast, neither Mg^{2+} nor $Cu(II)$ ions affected the absorption spectrum of Ret-P in Triton X-100 micellar solution (results not shown).

Effect of bivalent cations on Ret-P-Man synthesis

Since the $Mn(II)$ ion is known to be required for Ret-P-Man synthesis in microsomal membranes (Shidoji & De Luca, 1981), we investigated the activity of other bivalent cations to stimulate

Fig. 6. The u.v.-absorption spectrum of Ret-P (8 $\mu g/ml$) in BSA solution (1 mg/ml) at $Mn(II)$ concentrations of (A) zero, (B) 0.5 mM and (C) 1 mM

absorption spectrum of exogenously added Ret-P (25 μM) in a rat liver microsomal suspension (0.5 mg of protein/ml) containing 30 mM-Tris/HCl (pH 8) and 8 mM-NaF

Ret-*P*-Man synthesis at 5 mM concentration in the presence or in the absence of Triton X-100. Under either condition, Co(II), Mn(II) and Mg(II) were active, whereas Cr(II), Fe(II), Zn(II), Cu(II) and Ca²⁺ were without activity (Table 1). When the metal ions were studied at 2.5 mM, similar results were obtained except that MgCl₂ was less active than MnCl₂ (Table 1). Under these conditions we altered the order of addition so that the BSA was the last component added after the metal and Ret-*P* had formed the 'metachromatic complex'. As shown in Table 1 this alteration in the sequence of addition caused a 73% inhibition in Ret-*P*-Man synthesis in the presence of Co(II) and a 76% inhibition in the presence of Mn(II), whereas only 8.6% inhibition was observed in the presence of MgCl₂, which does not form the metachromatic complex. A lesser inhibition (from 840 to 520 c.p.m./mg per 15 min) in Ret-*P*-Man synthesis from endogenous Ret-*P* was found when BSA was added after the Mn(II).

Discussion

In the present paper we have described for the first time a profound spectral change of Ret-*P* induced by metal ions Mn(II), Ca²⁺, Zn(II), Fe(II), Cr(II) and Co(II) in Triton X-100 micelles. The Mn(II)-induced spectral changes ('metachromasy') of Ret-*P* were also observed either in the system of DPPC liposomes or in rat liver microsomes and they were reversible by EDTA.

The physical state giving rise to 'metachromasy' is not necessary for the activity of Ret-*P* as mannosyl acceptor, as shown by the fact that Mg²⁺, a metal ion without activity in inducing metachromasy of Ret-*P*, is active in Ret-*P*-Man synthesis and that Ca²⁺, Zn(II), Fe(II) and Cr(II), all active in inducing metachromasy, are without activity in Ret-*P*-Man synthesis. On the contrary, if the formation of the complex between Ret-*P* and the metal is allowed by adding BSA to the assay mixture after the metal ion, an inhibition of over 70% in the synthesis of Ret-*P*-Man is observed, possibly because Ret-*P* is no longer available for mannosylation, even at relatively low concentrations of the metal (2.5 mM). Ret-*P*-Man synthesis in incubations containing MgCl₂, which does not form the metachromatic complex, is not affected by the sequence of addition of the metal and the BSA in the reaction mixture, thus suggesting that formation of the 'metachromatic complex' renders the Ret-*P* unavailable for mannosylation.

From the e.s.r. data it can be suggested that the Mn(II)-induced spectral changes of Ret-*P* can be caused by either binding of Mn(II) to the phosphate group, which affects the electronic state of the chromophore indirectly through the phosphate moiety, or chelation of Mn(II) between two phosphate groups of Ret-*P*, allowing electronic interaction

between two chromophores. The first possibility can be dismissed, because no such marked spectral changes as shown in Fig. 1 have ever been observed due to phosphorylation of the terminal hydroxy group of retinol or esterification with fatty acids (Schwieter & Isler, 1967). Moreover, it is unlikely that the effect of polylysine on Ret-*P* can be explained by this mechanism. On the other hand, it is well known that a dimerization in which chromophores are stacked with a nearly parallel or anti-parallel array of their transition moments gives rise to spectral splitting on both sides of the monomer band in such a way that the intensity of the splitting band at the shorter wavelength is much stronger than that at the longer wavelength (Cantor & Schimmel, 1980). It should be noted here that two isosbestic points (at 303 nm and 368 nm) appear in the presence of Mn(II) ions as shown also in Fig. 1. This may indicate the existence of an equilibrium between the Mn(II)-bound monomer and a possible Mn(II)-bound dimer of Ret-*P* in Triton X-100 micelles.

Therefore, consideration should be given to the possibility that the availability of Ret-*P* for Ret-*P*-Man synthesis *in vivo* may depend on the balance between proteins such as BSA and the concentration of bivalent cations or polyamines in the proximity of the mannosyltransferase and Ret-*P* in the membrane.

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