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Hypothesis

Mechanism of pH-induced release of retinol from retinol-binding protein

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A hypothesis is proposed explaining the mechanism of pH-induced release of retinol from retinol-binding protein (RBP). A number of conservative positively charged side chains located on the retinol-binding face of the RBP molecule are involved in salt bridges with conservative negatively charged groups. At low pH these salt bridges are broken and the retinol-binding face of RBP holds from 8 to 12 positively charged groups, which can ensure a proper orientation of the RBP molecule relative to a negatively charged membrane, facilitating the release of retinol. The disruption of salt bridges and the electrostatic repulsion of positive charges can soften the structure of the molecule near the entrance to the retinol-binding pocket, which can trigger both the release of retinol and the transition of RBP to the molten globule state.

Retinol-binding protein; Retinol; Molten globule state; pH-induced release

1. INTRODUCTION

The target transport of non-polar ligands by protein carriers to membranes or membrane receptors involves the release of these ligands on or near membranes. These hydrophobic ligands are often bound inside a deep pocket, as in the case, e.g. of retinol-binding protein (RBP) [1–4]. Here the release of a hydrophobic ligand may be connected with a jump over a large potential barrier by strong interactions between a ligand and a protein moiety inside a pocket.

Retinol can be completely released from RBP in vitro by treatment with organic solvents [5], heating [6–8] or low pH [8]. It has been shown that the release of retinol is strongly accelerated by a decrease of pH in the presence of liposomes [9] as well as in their absence [8]. It has been shown also [8] that both *apo* and *holo*RBP (i.e. respectively, RBP without and with retinol) can be transformed at low pH into the molten globule state [10–13] which is the more or less typical state of globular proteins under mild denaturating conditions [13,14], and it has been suggested [15] and shown experimentally [16–18] to be involved in protein translocation or insertion into membranes.

In this paper we shall consider the surface of RBP near the hole of the retinol-binding cavity and show that the change of the pattern of charges at acidic pH may both facilitate RBP–membrane interaction and serve as a trigger mechanism for the release of retinol.

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2. METHODS

Patterns of charged groups have been studied using X-ray atomic coordinates of human *holo*RBP in the orthorhombic crystal form (resolution 1.9 Å) [2], human *holo* and *apo*RBP* in the trigonal form (resolution 2.5 Å) [3] and bovine *holo* and *apo*RBP (resolution 1.9 and 1.7 Å, respectively) [4]. The analysis was performed by means of program WHAT IF [19] and FRODO [20] using an Evans & Sutherland graphics system at the Department of Protein Design of EMBL (Heidelberg, Germany) and at the Department of Organic Chemistry of Padua University (Italy). The criterion for formation of a salt bridge was chosen as a distance <4 Å between one of the N-atoms of ε-NH_3^+-groups of Lys or guanidine groups of Arg and one of the O-atoms of COO^--groups of Asp or Glu.

Invariant or conservative charged groups were established by comparison of the amino acid sequences of human [21], bovine [22], rat, rabbit [23], *X. laevis* [24], and trout I and II [25] RBPs.

3. PATTERNS OF POSITIVE AND NEGATIVE CHARGES OF RETINOL-BINDING PROTEIN: SALT BRIDGES AT NEUTRAL pH AND POSITIVELY CHARGED CLUSTER AT ACID pH

The RBP molecule is a single domain protein, that encapsulates retinol in a cavity formed by an 8-stranded

*In this paper we will call *apo* the unliganded protein, that is the protein lacking retinol, independent of the method used to extract the vitamin.

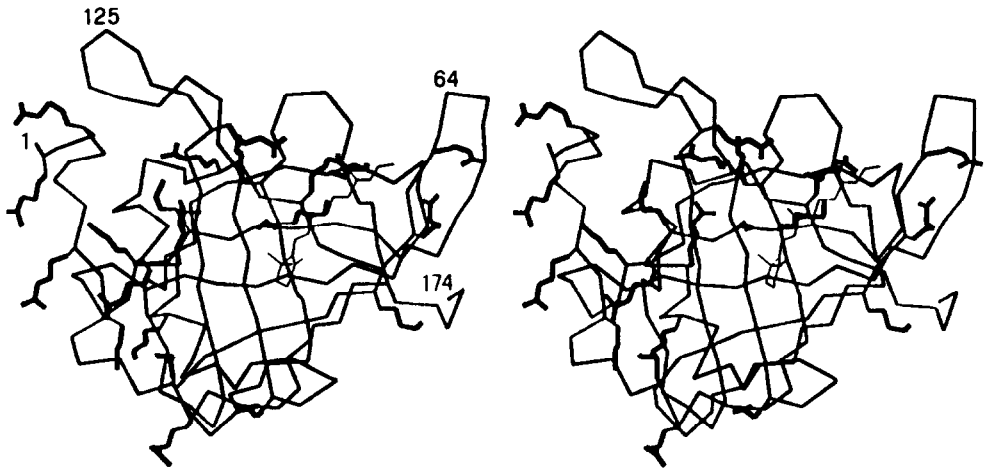

Fig. 1. Stereo drawing of positively charged side chains of human *holo*RBP [2], which remain positively charged in all the other known sequences [22–25]. The human protein in the trigonal crystal form [3] and bovine RBP [4] are very similar. The retinol molecule is shown by thin lines.

β-barrel [2–4]. The protein presents a quite large surface, formed (in notation of [2]) mainly by hairpins $\beta_A\beta_B$, $\beta_C\beta_D$, $\beta_E\beta_F$, $\beta_G\beta_H$, and by portions of strands F (residues from 100 to 103), G (from 121 to 123), H (from 129 to 131), of the C-terminal chain (from 160 to 166) and of the N-terminus (from 3 to 8) corresponding to the entrance of the retinol-binding cavity. This surface will be referred to as the face of the retinol-binding pocket. There are 13 positively charged and 10 negatively charged residues which are conserved in all known RBP sequences. Among the 13 positive side chains of human RBP, 8 are located on the surface containing the entrance of the retinol-binding cavity, while the other 5 are scattered on the rest of the protein surface (Fig. 1).

The pattern of charged side chains on the face of the retinol-binding pocket of human RBP, shown in Fig. 1, is schematically presented in Fig. 2. It contains 8 invariant or conservative positively charged residues (Lys-29$^+$, Lys-30$^+$ in β_A, Arg-60$^+$ in β_C, Lys-85$^+$, Lys/Arg-87$^+$, Lys/Arg-89$^+$ in β_E, Arg-121$^+$ in β_G and Arg-166$^+$ in the C-terminal strand) and 8 invariant or conservative negatively charged residues (Asp-31$^-$, in the loop $\beta_A\beta_B$, Asp-39$^-$ in β_B, Asp/Glu-68$^-$ in β_D, Asp-82$^-$ in the loop β_D-β_E, Asp-102$^-$ and Asp-103$^-$ in β_F, Asp-126$^-$ in the loop $\beta_G\beta_H$ and Asp-131$^-$ in β_H). Three of these conservative positive charges (Lys-29$^+$, Arg-60$^+$, Arg-121$^+$) and five conservative negative charges (Asp-31$^-$, Asp-39$^-$, Asp/Glu-68$^-$, Asp-102$^-$ and Asp-131$^-$) form the immediate surroundings of the entrance of the retinol-binding cavity.

A striking feature of this pattern is that *four conservative positively charged side chains (including the three which surround the hole) are involved in salt bridges with side chains of conservative negative (mostly aspartic acid) residues*. These four salt bridges, listed in Table I, are established from X-ray coordinates of human *holo* and *apo*RBP [2,3] and bovine *holo* and *apo*RBP [4]. In addi-

Table I

Conservative salt bridges near the hole of the retinol-binding pocket in RBPs

Salt bridges	$N^+...O^-$ distances (Å)				
	Human *holo*($P2_12_12_1$) [2]	Human *holo*(R3) [3]	Human *apo*(R3) [3]	Bovine *holo* [4]	Bovine *apo* [4]
Lys-29$^+$...Asp-31$^-$	2.94	2.54	2.72	2.78	3.86*
Arg-60$^+$...Asp-39$^-$	3.17	2.95	2.94	2.78	2.82
Arg-60$^+$...Asp/Glu-68$^-$	2.94	3.23	3.31	3.17	2.71
Lys/Arg-87$^+$...Asp-103$^-$	2.95	3.21	3.35	3.04	3.40
Arg-121$^+$...Asp-102$^-$	2.97	2.62	3.13	2.89	3.03

*In bovine *apo*RBP the side chain of Lys-29 makes an interaction with a polar group of a solvent molecule that substitutes the retinol inside the cavity.

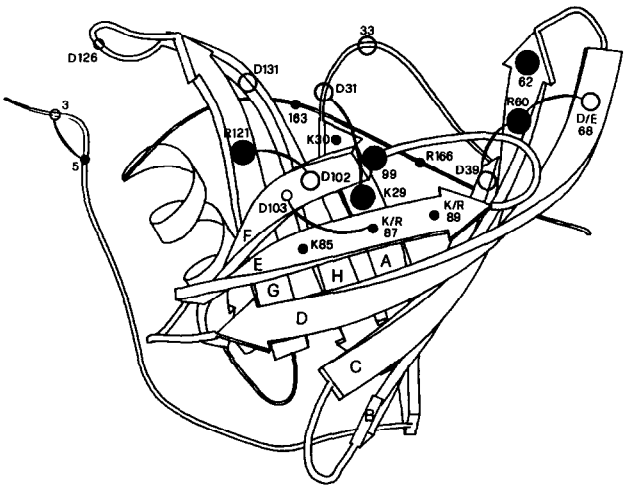

Fig. 2. Schematic drawing of the folding pattern of RBP, with charges on the face of the entrance to the retinol-binding cavity. β-Strands are named from A to H following [2]. Side chains which are positively and negatively charged in all mammalian RBPs are shown by black dots and open circles, respectively. If a residue is conserved also in all the other species for which the amino acid sequence is known, its type is indicated by the one-letter code; if not, only the number of the residue is indicated. Side chains forming salt bridges are linked.

tion, invariant Lys-85$^+$, although not satisfying our criteria for the formation of a salt bridge, is very close to invariant Asp-82$^-$ (from 4.04 to 4.61 Å in the different structures). These salt bridges contribute to the stabilization of the native 3D structure of RBP. Especially important is salt bridge Arg-121$^+$...Asp-102$^-$ which connects the top of strand β_G with strand β_F and contributes to screening the hole from solvent [2].

This picture is not changed by the pattern of non-conservative charged residues: in mammalian RBPs and in *X. laevis* there are two other positive charges (Arg-62$^+$ and Lys/Arg-99$^+$) and one negative charge (Glu-33$^-$) around the hole. In addition, almost all non-trout RBPs have two positive charges (Arg-5$^+$ and Arg-163$^+$) which belong to the same surface but are not in the immediate surrounding of the hole; they are involved in the salt bridges Arg-5$^+$...Asp-3$^-$ and Arg-163$^+$...Glu-157$^-$. In trout, residues 62 and 33 are hydrophobic (Ile and Val, respectively), 5 is Gln, 163 is Gly. Residue 99 is Thr or Ser in trout I and II, respectively.

The situation is quite different at acidic pH, when aspartic and glutamic acid residues shift towards neutrality. Salt bridges may decrease apparent p*K*s of these residues relative to their normal values (about 4.5), but at a pH well below 4.5 all negative charges should disappear, which leads to the disruption of all salt bridges and to a large change in the whole network of charges. At low pH, the face of the retinol-binding pocket is covered with 8 conservative positively charged groups (12 in mammalians RBPs), 4 of which (5 in mammals) were involved in salt bridges at neutral pH. The retinol-binding pocket is now surrounded by a halo of three invariant positively charged group (Lys-29$^+$, Arg-60$^+$ and Arg-121$^+$). Fig. 3 summarizes schematically the dramatic change that affects the retinol-binding face and the surrounding of the hole during a decrease in pH from neutral to acid values.

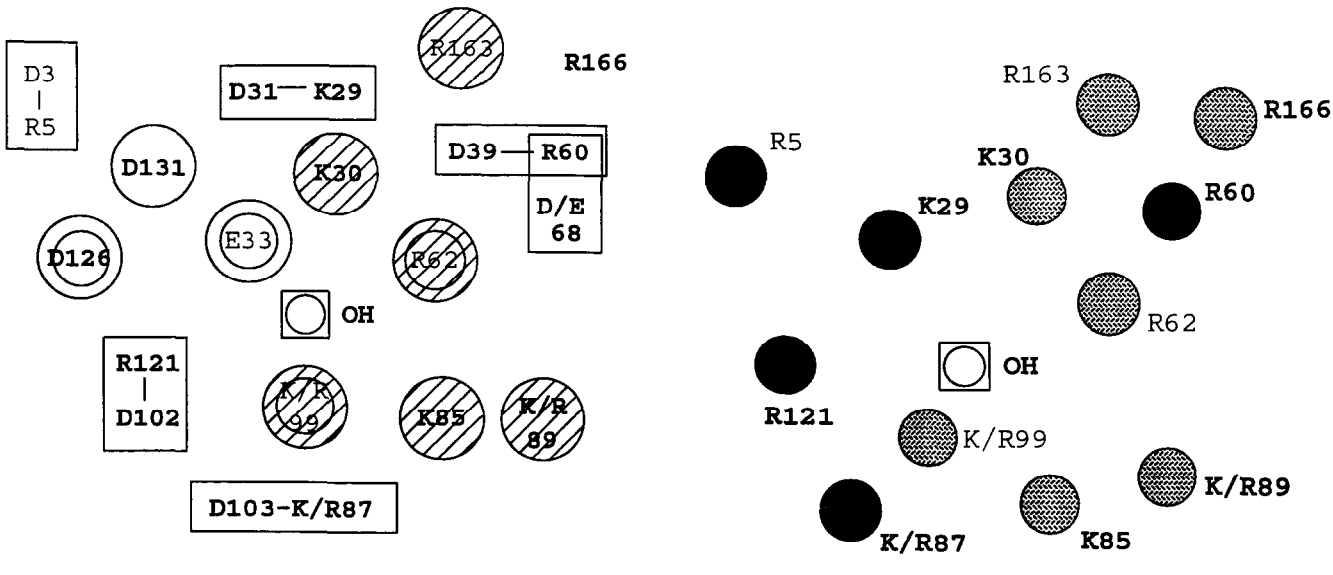

Fig. 3. A scheme of the immediate surrounding of the entrance to the retinol-binding pocket of RBP at neutral (a) and acid (b) pH. Conservative charges are shown by bold letters, salt bridges by rectangles. Positive charges not involved in salt bridges are darkened. OH indicates the position of retinol. Residues marked by a double line in (a) are situated above the plane where salt bridges are placed. Black dots in (b) mark positive charges which have been involved in salt bridges at neutral pH.

5. DISCUSSION

The formation of a large positively charged cluster on the retinol-binding pocket surface may lead to a proper orientation of the RBP molecule relative to a negatively charged membrane, facilitating targeted release of retinol. Moreover, the disruption of 4 salt bridges in the immediate environment of the hole can soften structures and change the relative positions of turns and loops surrounding it, which can further facilitate the release of retinol. In addition, the disruption of salt bridge Arg-121$^+$...Asp-102$^-$ leads to a partial opening of the hole (see above), while an electrostatic repulsion between positively charged groups surrounding the hole can lead to an additional opening of it. Finally, His-104, which is the only ionizable residue inside the cavity, could also play a role in the mechanism of retinol release.

As a result, the low pH can lead both to favourable electrostatic interactions of the retinol-binding surface with a negatively charged membrane, and to a local opening of the hole, facilitating targeted release of retinol from the native structure of RBP. This release may lead to the additional destabilization of the structure of RBP and trigger the transition of *apo*RBP to the molten globule state.

It is important to mention that a strong negative electrostatic potential of membrane can lead to a local decrease of pH to acid values even when the bulk pH value is neutral. The theoretical estimate of this shift is as large as 2.7 pH units, while the experiment for negatively charged liposomes gives 1.6 units [16]. Therefore, we can expect [8] that a pH-induced mechanism for the release of retinol from RBP can operate near the membrane surface even at neutral or almost neutral bulk pH.

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