pH-induced transition and Zn^{2+}-binding properties of bovine prolactin[1]

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Received 16 January 1997; revised version received 17 February 1997

Abstract A pH-induced conformational transition was found in bovine prolactin within the physiologically significant pH region from 6.5 to 8.5. The thermal stability of prolactin at pH 6.5 is essentially higher than at pH 8.5. Bovine prolactin binds zinc ions with an apparent association constant of 2×10^5 M^{-1} at pH 6.5 and 1×10^4 M^{-1} at pH 8.5. The pH dependence of both thermal stability and zinc binding surrounding the pK_a of histidine suggests that these residues plays a key role in the structural integrity of bovine prolactin.

Key words: Prolactin; Zinc binding; Intrinsic fluorescence; pH-induced transition

1. Introduction

Prolactin (PRL) is a pituitary hormone which regulates numerous physiological functions, including lactation and cell division [1]. The prolactin receptor (PRLr), located on the cell membrane, is activated upon formation of the 1 PRL:2 PRLr complex [2]. The formation of this heterogeneous trimeric complex activates the intracellular portion of the prolactin receptor to associate and activate Janus and other kinases [3]. PRL is closely related to growth hormone (GH) [4]. Primate GHs, including human GH, are unique in that they bind and activate both the human GHr and PRLr. A strong Zn^{2+} dependence of human GH binding to site 1 of the human PRLr has been demonstrated [5,6]. The crystallographic structure of human GH bound to a single extracellular domain of human PRLr revealed the location of a Zn-binding site on the hormone-receptor interface, formed by His-18 and Glu-174 from human GH and His-218 and Asp-217 from PRLr [7]. At the same time, no Zn^{2+} activation of PRL binding to the PRLr was found, although PRL has the same Zn^{2+}-binding determinants.

Zinc binds to human GH in the absence of receptor, causing its dimerization [8] in a highly concentration-dependent fashion. The same residues (i.e. His-18, His-21, and Glu-174) were important for the zinc-dependent dimerization as the zinc-mediated binding of human GH to PRLr [5].

Zinc may play a role in the formation of PRL storage granules in the pituitary where storage of PRL in an osmotically neutral and biologically inactive form is desirable. Zinc is present in high concentrations in pituitary PRL granules [9], and a possible role of zinc in PRL storage/release has long been recognized [10]. Rat pituitary cells grown in vitro do not store PRL when zinc is removed from the media [11]. Similarly, humans with zinc deficiency have high serum PRL concentrations [12], suggesting a zinc requirement for pituitary storage.

Until recently there has been no direct evidence for zinc binding to PRL. Recently, kallikrein-mediated cleavage of PRL was shown to be reduced in the presence of zinc [13], although binding constants or the effects of zinc binding on protein conformation were not determined.

Very recent work in our laboratories demonstrated that PRL phosphorylation occurs in membrane fractions of the bovine pituitary in a zinc-dependent manner (Wicks and Brooks, in preparation). Elimination of the putative zinc binding coordinant by mutation of His-27 to Ala (H27A bovine PRL) resulted in a reduction of zinc-dependent phosphorylation (Wicks and Brooks, unpublished data). These results suggest that zinc binding to bovine PRL is required to provide an appropriate substrate conformation for kinase-dependent phosphorylation.

In the present study, we used intrinsic protein fluorescence to describe a pH-induced structural transition in bovine PRL within the physiologically significant pH region from 6.5 to 8.5 and compared thermostability and zinc-binding constants of the protein at pH values on either side of this transition.

2. Materials and methods

Bovine prolactin (bPRL) was isolated and purified as previously described [14]. The protein was in the non-phosphorylated form. Tris, Bis-Tris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane), and HEPES were from Sigma Chemical Co. EGTA was from Serva. $ZnSO_4$ was from Reakhim (USSR). The salt was dried for 24 h before stock solution preparation. Only distilled deionized water was used in this work. All other reagents were of the highest grade available and used without further purification.

Protein concentration was estimated spectrophotometrically using an extinction coefficient $E_{1\%,\ 280\ nm} = 9.09$ [15]. Fluorescence measurements were carried out on a laboratory-made spectrofluorimeter [16]. All spectra were corrected for the spectral sensitivity of the instrument. Temperature was monitored inside the sample cell with a copper-constantan thermocouple. Samples were equilibrated at each temperature for 5 min prior to spectral measurements. The temperature dependence of fluorescence parameters was analyzed as previously described [17]. The apparent binding constants for Zn^{2+} were calculated by fitting the spectrofluorimetric titration data to the specific binding scheme [18] using non-linear regression analysis [19,20]. The binding scheme was chosen on the 'simplest best fit' basics, also taking into consideration fluorescence phase plots [21]. The quality of the fit was judged by a randomness of distribution of residuals. The accuracy

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[1] Supported by the National Institutes of Health (DK 42604 and DK 01989)

PII S0014-5793(97)00203-2

of the evaluation of the binding constants was approximately one half order of magnitude.

Due to the slow solubility of bovine PRL, it was first dissolved in the buffer at pH 6.5 and then pH was adjusted to the desired value. The absorbance of the sample protein was typically between 0.02 to 0.07.

3. Results and discussion

Spectrofluorimetric NaCl-titration of bPRL up to a concentration of 1 M showed no ionic strength dependence of tryptophan fluorescence parameters (data are not shown), therefore, all subsequent experiments were performed at low ionic strength (i.e. in 10–20 mM buffer).

Fig. 1 depicts the pH dependence of tryptophan fluorescence parameters (emission maximum position and relative fluorescence quantum yield) for bovine PRL in the region from 2 to 12. The transition (increase in relative fluorescence quantum yield and 2 nm red spectral shift) with increase in proton concentration (within the pH region from 5 to 3) reflects structural changes induced by protonation of carboxylates of aspartic and glutamic acid. The decrease in fluorescence quantum yield and the 10 nm red spectral shift at pH 9–12 are caused by denaturing conformation changes caused by deprotonation of amino groups of lysines and phenolic groups of tyrosines. These data are in agreement with the results of Edelhoch and colleagues [22,23]. The most interesting feature of the pH dependence of bovine prolactin is a transition (a decrease in fluorescence quantum yield) within the physiologically significant pH region from 6.5 to 8.5 (Fig. 1A), which was not reported earlier. Reductions in the proton concentration in this region deprotonate the imidazole group of histidine leaving the protein with a greater net negative charge. The changes in fluorescence indicate alterations in tryptophan environment, a measure of structural change and are indicative of pH-induced transition between two structural conformations.

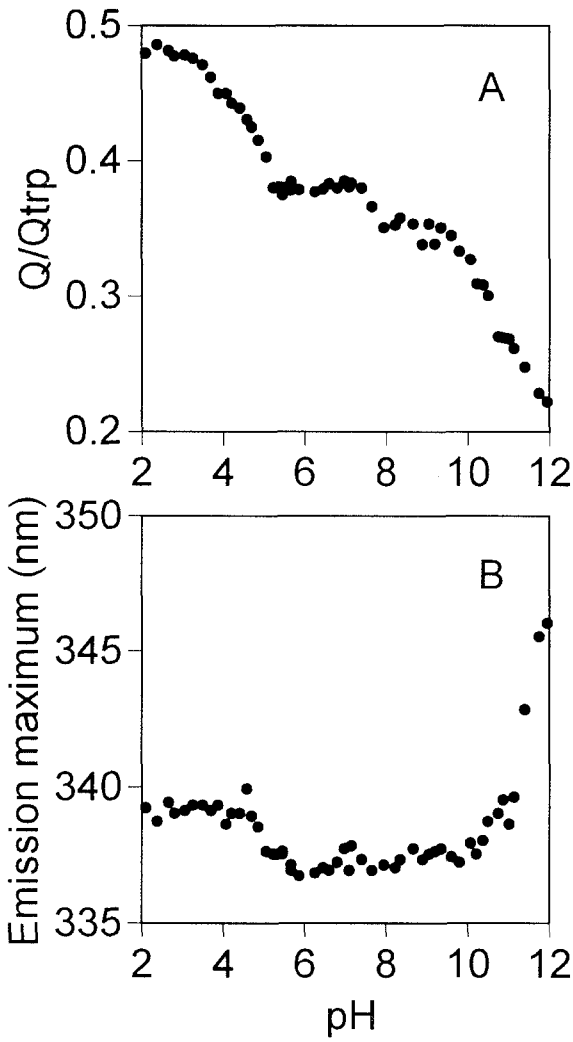

Fig. 1. pH dependence of bovine prolactin fluorescence. (A) Fluorescence quantum yield; (B) fluorescence maximum position. Excitation at 280 nm. Sample prepared in 20 mM Bis-Tris propane buffer. Temperature: 21°C.

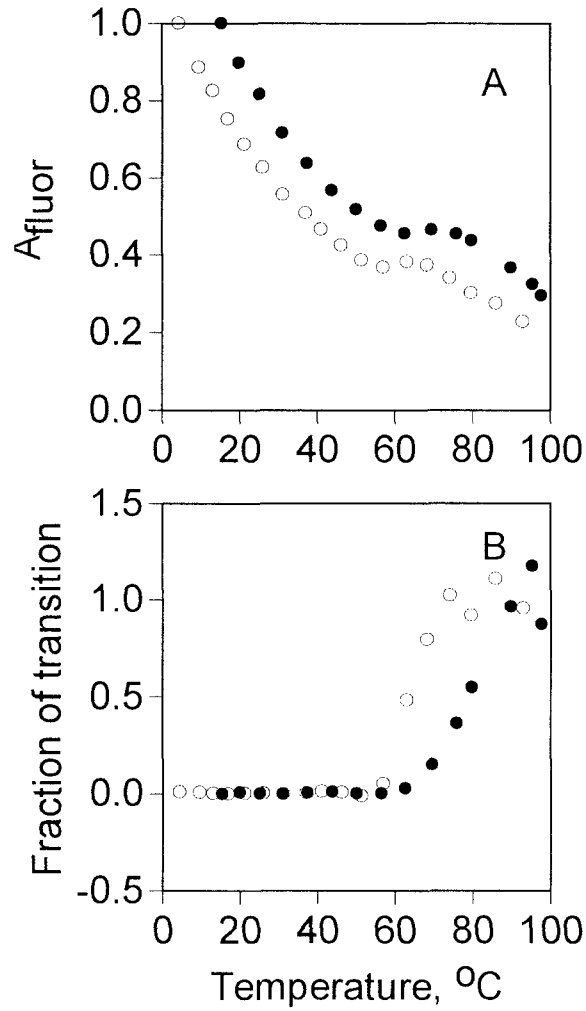

Fig. 2. Temperature dependence of bovine prolactin fluorescence. (A) Area under fluorescence spectra; (B) fraction of conversion, calculated from the fluorescence intensity data at 350 nm. Samples prepared in 10 mM HEPES, pH 6.5 (●) and 8.5 (○).

We next compared the thermal stabilities and zinc binding of bovine PRL in these two pH-induced conformations. Fig. 2 shows fluorescence data during thermal denaturation of bovine PRL. Fig. 2A depicts the the temperature dependence of relative fluorescence intensity and Fig. 2B demonstrates the temperature dependence of the fraction of conversion from the native to thermally denatured state of bPRL which was

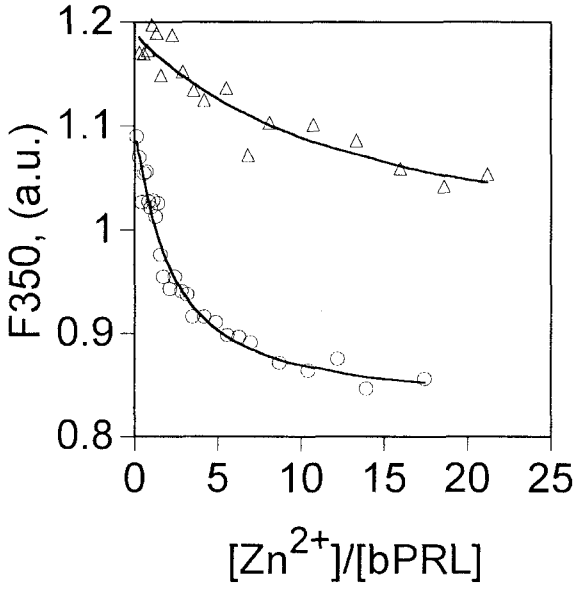

Fig. 3. Spectrofluorimetric titration of bovine prolactin with zinc at pH 6.5 (○) or 8.5 (△). Fluorescence intensity was monitored at 350 nm, excitation at 280 nm. Conditions were: 10 mM HEPES, 20°C. Points represent experimental data, while the curves denote the calculated fit.

derived from the data presented in Fig. 2A [17]. The denaturation (60–80°C region) of the protein leads to a decrease in intramolecular fluorescence quenching (slight increase in fluorescence yield), similar to acid denaturation. Fig. 2B shows that bPRL at pH 6.5 is essentially more stable than at 8.5 (midpoints of the transitions are 78 and 63°C, respectively). The pH-induced decrease in thermal stability of bPRL can be explained by disruption of some intramolecular bonds where histidines act as proton donors. The fluorescence maxima do not change significantly within the 20–90°C region: 336 to 338 nm at pH 6.5 and 336 to 340 nm at pH 8.5 (data are not shown).

Bovine PRL was titrated with increasing concentrations of Zn^{2+} and the fluorescence intensity at 350 nm measured at pH 6.5 and 8.5 (Fig. 3). Gradual increases in Zn^{2+} concentration resulted in a decrease in relative fluorescence intensity without any changes in maximal spectrum position. The fluorescence changes are caused by Zn^{2+} binding to the protein which results in a change in conformation detected by the environment surrounding the two tryptophans and expressed as a change in the intensity. It is evident from Fig. 3 that the reduction of fluorescence intensity occurs at lower Zn^{2+} concentration at pH 6.5. The curves are best fit by the simplest one-site binding scheme: bPRL+Zn^{2+} ⇆ bPRL-Zn^{2+}. The association constants which provide the best fit are 2×10^5 M^{-1} at pH 6.5 and 1×10^4 M^{-1} at pH 8.5. The decrease in affinity of zinc binding in the pH region from 6.5 to 8.5 suggests an involvement of histidines in the pH-induced transition.

One might expect an increase in the binding constant with an increase in pH if histidines take part in coordination of the metal ion. However, in our case, a decrease in the binding constant with the increase in pH is observed. Two interpretations of the data are possible. First, the six histidines which are not involved in Zn^{2+} binding are deprotonated with increasing pH and induce a conformation change (described in Fig. 1) which more than compensate for any increase in Zn^{2+} affinity produced by the deprotonation of His-27. The second interpretation is that His-27 is not involved in Zn^{2+} binding to bovine PRL. It should be noted that this second interpretation is in contradiction with the existence of His residues in the zinc-binding site of human GH [5,7,8]. The second interpretation also contradicts our work involving the Zn^{2+}-dependent phosphorylation of bovine PRL in pituitary secretory granules (Wicks and Brooks, in preparation). In those studies mutation of His-27 to Ala severely reduced zinc-dependent phosphorylation, suggesting His-27 is one site facilitating Zn^{2+} binding in bovine PRL.

In the pituitary gland prolactin is synthesized in the rough endoplasmic reticulum and processed through the Golgi to either be secreted or stored in condensed granules where PRL concentrations reach several millimolar [24]. The vesicular pH drops during granule formation to values less than 6. In addition, Zn^{2+} is present in millimolar concentrations [13]. Under these conditions the zinc-bound form of bovine PRL will predominate. In contrast, when bovine PRL is released into the blood the pH is commonly between 7.2 and 7.6 [25], bovine PRL will be found in the low nanomolar range and the free Zn^{2+} concentration will be less than one micromolar [12]. Under these conditions the portion of zinc-bound bovine PRL will be less than 10% of the total.

This study demonstrates Zn^{2+}-binding by bovine PRL, a pH-induced transition in the region between 6.5 and 8.5, and involvement of histidine in the overall structure of bovine prolactin and its ability to bind Zn^{2+}.

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