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Structure and stability of recombinant bovine odorant-binding protein: II. Unfolding of the monomeric forms

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In a family of monomeric odorant-binding proteins (OBPs), bovine OBP (bOBP), that lacks conserved disulfide bond found in other OBPs, occupies unique niche because of its ability to form domain-swapped dimers. In this study, we analyzed conformational stabilities of the recombinant bOBP and its monomeric variants, the bOBP-Gly121+ mutant containing an additional glycine residue after the residue 121 of the bOBP, and the GCC-bOBP mutant obtained from the bOBP-Gly121+ form by introduction of the Trp64Cys/His155Cys double mutation to restore the canonical disulfide bond. We also analyzed the effect of the natural ligand binding on the conformational stabilities of these bOBP variants. Our data are consistent with the conclusion that the unfolding-refolding pathways of the recombinant bOBP and its mutant monomeric forms bOBP-Gly121+ and GCC-bOBP are similar and do not depend on the oligomeric status of the protein. This clearly shows that the information on the unfolding-refolding mechanism is encoded in the structure of the bOBP monomers. However, the process of the bOBP unfolding is significantly complicated by the formation of the domain-swapped dimer, and the rates of the unfolding-refolding reactions essentially depend on the conditions in which the protein is located.

1 **Structure and Stability of Recombinant Bovine Odorant-**

2 **Binding Protein: II. Unfolding of the Monomeric Forms**

3

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19 **Running title:** Unfolding of the monomeric bOBP variants

21 **ABSTRACT**

22 In a family of monomeric odorant-binding proteins (OBPs), bovine OBP (bOBP), that lacks
23 conserved disulfide bond found in other OBPs, occupies unique niche because of its ability to
24 form domain-swapped dimers. In this study, we analyzed conformational stabilities of the
25 recombinant bOBP and its monomeric variants, the bOBP-Gly121+ mutant containing an
26 additional glycine residue after the residue 121 of the bOBP, and the GCC-bOBP mutant
27 obtained from the bOBP-Gly121+ form by introduction of the Trp64Cys/His155Cys double
28 mutation to restore the canonical disulfide bond. We also analyzed the effect of the natural
29 ligand binding on the conformational stabilities of these bOBP variants. Our data are consistent
30 with the conclusion that the unfolding-refolding pathways of the recombinant bOBP and its
31 mutant monomeric forms bOBP-Gly121+ and GCC-bOBP are similar and do not depend on the
32 oligomeric status of the protein. This clearly shows that the information on the unfolding-
33 refolding mechanism is encoded in the structure of the bOBP monomers. However, the process
34 of the bOBP unfolding is significantly complicated by the formation of the domain-swapped
35 dimer, and the rates of the unfolding-refolding reactions essentially depend on the conditions in
36 which the protein is located.

37

38 **Key words:** odorant-binding protein; domain swapping; disulfide bond; unfolding-refolding
39 reaction; ligand binding; conformational stability

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41

43 INTRODUCTION

44 Odorant binding proteins (OBPs) are important components of olfactory apparatus in
45 vertebrates where they play a specific role in olfaction by interacting directly with odorants
46 (Xu et al. 2005). OBPs constitute a class of small extracellular proteins in the
47 chemosensory systems of most terrestrial species ranging from drosophila to human. In
48 mammals, OBPs are found at high concentrations (~10 mM) in nasal mucosa of cow
49 (Bignetti et al. 1985; Pelosi et al. 1982), rat (Pevsner et al. 1985), rabbit (Dal Monte et al.
50 1991), pig (Dal Monte et al. 1991), dog (D'Auria et al. 2006), and humans (Briand et al.
51 2002). Although they bind different kinds of small and hydrophobic odorant molecules
52 (typically with the affinities in the micromolar range, their inability to discriminate
53 different chemical classes of these molecules suggests that OBPs cannot serve as olfactory
54 receptors (Boudjelal et al. 1996). The precise biological functions of mammalian OBPs are
55 not known as of yet, but it was hypothesized that these proteins can be involved in transport
56 of hydrophobic odorants across the aqueous mucus layer to access the olfactory receptors,
57 or might be involved in the termination of the olfactory signal by removing odorants from
58 the receptors after their stimulation (Bignetti et al. 1987; Pevsner & Snyder 1990).

59 OBPs constitute a sub-class of lipocalins, which are small extracellular proteins found
60 in gram negative bacteria, plants, invertebrates, and vertebrates. Although lipocalins are
61 known to share limited regions of sequence homology, they do have a common tertiary
62 structure architecture (Flower et al. 2000; Grzyb et al. 2006). The characteristic structural
63 signature of the lipocalin family is a β -barrel composed by a 9-stranded anti-parallel β -
64 sheet with an α -helical segment at the C-terminus (Flower et al. 2000). The internal cavity
65 of the lipocalin β -barrel is the binding site that can interact with the odorant molecules

66 belonging to different chemical classes (Vincent et al. 2000). Bovine OBP (bOBP) was the
67 first OBP for which crystal structure was solved, and the analysis of this structure revealed
68 that bOBP exists as a domain-swapped dimer (Bianchet et al. 1996; Tegoni et al. 1996).
69 This was in contrast to structures of other lipocalins, including the porcine OBP (pOBP)
70 (Spinelli et al. 1998), which are monomeric proteins. The ability of bOBP to form domain-
71 swapped dimers was explained by the absence of a glycine residue at the hinge region
72 linking the β -barrel to the α -helix, and by the lack of the disulfide bridge which is present
73 in all lipocalin sequences identified so far (Ramoni et al. 2002).

74 This work is dedicated to the analysis of the peculiarities of the GdnHCl-induced
75 unfolding – refolding reactions of the recombinant bOBP and its monomeric mutants,
76 bOBP-Gly121+ and bOBP-Gly121+/W64C/H155C (GCC-bOBP). It continues a series of
77 articles dedicated to the analysis of the effect of the environmental feature (including the
78 presence of crowding agents) on structural properties and conformational stability of
79 bOBP. The mutant protein bOBP-Gly121+ contains an extra glycine residue introduced
80 after the bOBP residue 121. This substitution was shown to promote monomerization of the
81 bOBP (Stephanenko et al. 2015) likely via increasing the mobility of the loop connecting α -
82 helix and 8th β -strand of the β -barrel. Substitutions of the residues Trp64 and His156 to
83 cysteines in bOBP-Gly121+ generate a mutant form GCC-bOBP, which is expected to have
84 stable monomeric structure due to the restoration of the disulfide bond typically seeing in
85 other OBPs (Ramoni et al. 2008). We also investigated the role of the natural ligand in the
86 stabilization of protein structure and looked at how the ligand binding affected the folding-
87 unfolding reaction of these proteins.

88

89 MATERIAL AND METHODS

90 *Materials*

91 GdnHCl (Nacalai Tesque, Japan), 1-octen-3-ol (OCT; Sigma-Aldrich, USA) and ANS
92 (ammonium salt of 8-anilinonaphtalene-1-sulfonic acid; Fluka, Switzerland) were used without
93 further purification. The protein concentration was 0.1 – 0.2 mg/ml. The OCT concentration was
94 10 mM. The experiments were performed in 20 mM Na-phosphate-buffered solution at pH 7.8.

95

96 *Gene expression and protein purification*

97 The plasmids pT7-7-bOBP which encodes bOBP and its mutant forms with a poly-
98 histidine tag were used to transform *Escherichia coli* BL21(DE3) host (Invitrogen) (Stepanenko
99 et al. 2014c). The protein expression was induced by incubating the cells with 0.3 mM of
100 isopropyl-beta-D-1-thiogalactopyranoside (IPTG; Fluka, Switzerland) for 24 h at 37 °C. The
101 recombinant protein was purified with Ni⁺-agarose packed in HisGraviTrap columns (GE
102 Healthcare, Sweden). The protein purity was determined through SDS-PAGE in 15%
103 polyacrylamide gel (Laemmli 1970).

104

105 *Fluorescence spectroscopy*

106 Fluorescence experiments were performed using a Cary Eclipse spectrofluorometer
107 (Varian, Australia) with microcells FLR (10 x 10 mm; Varian, Australia). Fluorescence lifetime
108 were measured using a “home built” spectrofluorometer with a nanosecond impulse (Stepanenko
109 et al. 2014a; Stepanenko et al. 2012; Turoverov et al. 1998) as well as micro-cells (101.016-QS 5
110 x 5 mm; Hellma, Germany). Tryptophan fluorescence in the protein was excited at the long-wave

111 absorption spectrum edge ($\lambda_{\text{ex}} = 297$ nm), wherein the tyrosine residue contribution to the bulk
112 protein fluorescence is negligible (Stepanenko et al. 2015). The fluorescence spectra position and
113 form were characterized using the parameter $A = I_{320}/I_{365}$, wherein I_{320} and I_{365} are the
114 fluorescence intensities at the emission wavelengths 320 and 365 nm, respectively (Turoverov &
115 Kuznetsova 2003). The values for parameter A and the fluorescence spectrum were corrected for
116 instrument sensitivity. The tryptophan fluorescence anisotropy was calculated using the
117 equation: $r = (I_V^V - GI_H^V)/(I_V^V + 2GI_H^V)$, wherein I_V^V and I_H^V are the vertical and horizontal
118 fluorescence intensity components upon excitement by vertically polarized light. G is the
119 relationship between the fluorescence intensity vertical and horizontal components upon
120 excitement by horizontally polarized light ($G = I_V^H / I_H^H$), $\lambda_{\text{em}} = 365$ nm (Turoverov et al. 1998).
121 The fluorescence intensity for the fluorescent dye ANS was recorded at $\lambda_{\text{em}} = 480$ nm ($\lambda_{\text{ex}} = 365$
122 nm).

123

124 *GdnHCl-induced unfolding*

125 Protein unfolding was initiated by manually mixing the protein solution (40 μL) with a
126 buffer solution (510 μL) that included the necessary GdnHCl concentration in the absence or
127 presence of a natural ligand, 1-Octen-3-ol (OCT). The GdnHCl concentration was determined by
128 the refraction coefficient using an Abbe refractometer (LOMO, Russia; (Pace 1986)). The
129 dependences of different fluorescent characteristics of the studied proteins on GdnHCl were
130 recorded following protein incubation in a solution with the appropriate denaturant concentration
131 at 4°C for different times (see in the text). The protein refolding was initiated by diluting the pre-
132 denatured protein (in 3.0 M GdnHCl, 40 μL) with the buffer or denaturant solutions at various

133 concentrations (510 μ L). The spectrofluorimeter was equipped with a thermostat that holds the
134 temperature constant at 23°C.

135

136 *Circular dichroism measurements*

137 The CD spectra were generated using a Jasco-810 spectropolarimeter (Jasco, Japan). Far-
138 UV CD spectra were recorded in a 1-mm path length cell from 260 nm to 190 nm with a 0.1 nm
139 step size. For the spectra, we generated 3 scans on average. The CD spectra for the appropriate
140 buffer solution were recorded and subtracted from the protein spectra.

141

142 *Gel filtration experiments*

143 We performed gel filtration experiments for recombinant bOBPwt and its mutant forms in
144 a buffered solution and with addition of GdnHCl using a Superdex-75 PC 3.2/30 column (GE
145 Healthcare, Sweden) and an AKTApurifier system (GE Healthcare, Sweden). The column was
146 equilibrated with the buffered solution or GdnHCl at the desired concentration, and 10 μ l of the
147 protein solution prepared under the same conditions was loaded on the pre-equilibrated column.
148 The change in hydrodynamic dimensions for the studied proteins was evaluated as a change in
149 the protein elution volume. Multiple proteins with known molecular masses (aprotinin (6.5 kDa),
150 ribonuclease (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa) and conalbumin (75
151 kDa), which are chromatography standards from GE Healthcare) were used to generate the
152 calibration curve.

153

154

155 RESULTS AND DISCUSSION

156 *Structural features of the monomeric and dimeric bOBPs*

157 Figure 1 compares structural features of the natural bOBP and its monomeric mutant GCC-
158 bOBP. Structures shown in Figures 1A and 1B indicate that the domain swapping has been
159 reverted in monomeric GCC-bOBP. Furthermore, Figure 1C represents the results of the multiple
160 structural alignment of the one of the monomers of the natural dimeric bOBP (blue structure),
161 monomeric mutant GCC-bOBP (red structure), and naturally monomeric pOBP (green structure)
162 and clearly shows that the monomeric GCC-bOBP has the overall fold almost identical to that of
163 the pOBP. Next, we analyzed how introduced mutations affected the structural flexibility of
164 bOBP utilizing the power of the FlexPred tool that rapidly predicts absolute per-residue
165 fluctuations from a three-dimensional structure of a query protein (Jamroz et al. 2012). Results of
166 this analysis are shown in Figure 1D which clearly indicates that the chains of the bOBP dimer,
167 chains of the naturally monomeric pOBP and the monomeric mutant GCC-bOBP are all
168 characterized by very similar structural flexibility. We also compared these structure-based
169 flexibility results with the bOBP propensity for intrinsic disorder evaluated by PONDR® VSL2B,
170 which is one of the more accurate stand-alone disorder predictors (Fan & Kurgan 2014; Peng et
171 al. 2005; Peng & Kurgan 2012). Figure 1D clearly shows that there is generally a very good
172 agreement between the structural flexibility calculated from the protein crystal structure and the
173 propensity of a protein for intrinsic disorder.

174

175 *Equilibrium unfolding of the recombinant bOBP in the presence of natural ligand*

176 Previously we have shown that the recombinant bOBP, unlike the natural bOBP extracted

177 from the tissues, represents a mixture of monomeric and dimeric forms suggesting that this
178 protein exists in a stable native-like state with a reduced dimerization capability (Stepanenko et
179 al. 2014c) (Table 1, Figure 2). It has been suggested that the recombinant form of bOBP is
180 characterized by the disturbed package of the α -helical region and some β -strands, which
181 prevents the formation of a native domain-swapped dimer (Stepanenko et al. 2014c). It is likely
182 that the dimer formation via the domain exchange mechanism is a rather complex process that
183 requires specific organization of the secondary and tertiary structure within the monomers.
184 Curiously, recombinant bOBP can form the compact dimeric state under the mild denaturing
185 conditions, namely, in the presence of 1.5 M guanidine hydrochloride (GdnHCl) (Stepanenko et
186 al. 2014c). This process requires bOBP restructuring and is accompanied by the formation of a
187 stable, more compact, intermediate state that is maximally populated at 0.5 M GdnHCl. The
188 increase of the GdnHCl concentration above 1.5 M induces cooperative unfolding of the
189 recombinant bOBP which is completed by ~ 3 M GdnHCl (Stepanenko et al. 2014c). The half-
190 transition point of this unfolding process at > 2 M GdnHCl indicates high conformational
191 stability of the recombinant bOBP (Stepanenko et al. 2014c), which is comparable with that of
192 the native (isolated from tissue) bOBP (Mazzini et al. 2002) and pOBP (Staiano et al. 2007;
193 Stepanenko et al. 2008) and is inherent to β -rich proteins (Stepanenko et al. 2012; Stepanenko et
194 al. 2013; Stepanenko et al. 2014b). We have also established that the unfolding of recombinant
195 protein is a completely reversible process, whereas the preceding process of the dimerization of
196 recombinant bOBP is the irreversible event (Stepanenko et al. 2014c).

197 To understand how interaction of the recombinant bOBP with its natural ligand, 1-Octen-3-
198 ol (OCT) affects this protein, we investigated structural properties and conformational stability
199 of the recombinant bOBP in the presence and absence of OCT. The formation of the protein-

200 ligand complex does not affect the oligomeric status of the protein, since according to the gel
201 filtration analysis the protein in the bOBP/OCT complex continue to exist as a mixture of
202 monomeric and dimeric molecules. However, both monomeric and dimeric forms of the protein
203 become more compact as a result of the OCT binding (Table 2, Figure 3). The GdnHCl-induced
204 unfolding of the bOBP/OCT complex is a rather slow process, since the equilibrium unfolding
205 curves are achieved after incubation of the bOBP/OCT in the presence of different
206 concentrations of the denaturing agent for more than 24 hours. However, the established
207 equilibrium was not affected by further incubation for up to 5 days (Figure 4).

208 Figure 4 shows that the complexity of the bOBP/OCT unfolding is clearly illustrated by the
209 complex shapes of the equilibrium dependencies of various characteristics of this complex on
210 GdnHCl concentration. This suggests the accumulation of several intermediate states, which are
211 similar to partially folded species found during the equilibrium unfolding of the recombinant
212 bOBP in the absence of OCT. However, compared to the bOBP alone, in the case of the
213 unfolding of bOBP/OCT complex, the accumulation of these intermediate states takes place at
214 higher denaturant concentrations. In fact, a more compact intermediate state of the bOBP/OCT
215 complex is formed in the concentration range of 0.26 - 1.0 M GdnHCl, whereas the transition of
216 the bOBP/OCT complex to the dimeric state occurs only at 2.0 M GdnHCl (Figure 3). Increasing
217 the GdnHCl concentration over 2.0 M leads to the cooperative unfolding of the bOBP/OCT
218 complex. In comparison with the unfolding of the recombinant bOBP alone, the unfolding of the
219 bOBP/OCT complex occurs in a narrow concentration range, and higher denaturant
220 concentrations are required for complete unfolding of this complex. All these data indicate that
221 formation of the bOBP/OCT complex leads to the substantial stabilization of the protein, but
222 does not affect its unfolding mechanism.

223 To better understand the GdnHCl-induced unfolding of the bOBP/OCT complex, we
224 analyzed the dependence of the 8-anilinonaphthalene-1-sulfonic acid (ANS, also called 1-
225 anilino-8-naphthalenesulfonate), fluorescence on the denaturant concentration. This hydrophobic
226 fluorescent probe is frequently used for the analysis of the presence of solvent-exposed
227 hydrophobic patches in a protein (Stryer 1965) and for the detection of accumulation of partially
228 folded intermediates during equilibrium and kinetic protein unfolding-refolding processes due
229 the ability of ANS to bind to such solvent-exposed hydrophobic patches (which are commonly
230 found in partially folded proteins) and due to the fact that this interaction can be easily detected
231 by the significant increase in the ANS fluorescence intensity and a characteristic blue-shift of its
232 fluorescence maximum (Semisotnov et al. 1991). The shape of the unfolding curve monitored by
233 the GdnHCl-induced changes in the fluorescence intensity of ANS added to the bOBP/OCT was
234 remarkably different from the unfolding curve measured for the recombinant bOBP alone. We
235 observe a smooth continuous decrease in the ANS fluorescence intensity at moderate GdnHCl
236 concentrations, with the ANS fluorescence intensity reaching zero at the denaturant
237 concentrations leading to the formation of the compact dimeric form of bOBP (Figure 4).

238 These observations suggest that ANS interacts with bOBP at sites close to and/or
239 overlapping with the ligand binding sites. Therefore, the formation of the bOBP/OCT complex
240 prevents ANS binding. Earlier analysis of the dimeric bOBP structure revealed the presence of
241 an additional ligand binding site at the interface between the monomeric subunits (Bianchet et al.
242 1996; Ikematsu et al. 2005; Pevsner et al. 1985). However, this inter-subunit binding site was
243 shown to be noticeably weaker than the major ligand binding site located within the β -barrel
244 (Bianchet et al. 1996; Ikematsu et al. 2005; Pevsner et al. 1985). Our data agree with the
245 presence of an additional ligand binding site in a protein. At the formation of the dimeric

246 bOBP/OCT complex with the native-like compactness at 2.0 M GdnHCl, this additional site is
247 occupied by the ligand, also preventing its interaction with ANS.

248 Moderate ANS fluorescence is detected in solutions containing less than 2 M GdnHCl; i.e.,
249 under conditions where the bOBP/OCT complex exists as a mixture of monomeric and dimeric
250 molecules, which are different from the native dimeric form of the bOBP. Under these
251 conditions, ANS fluorescence intensity in the presence of the bOBP/OCT complex is noticeably
252 lower than the ANS fluorescence recorded for the bOBP alone. These observations suggest that
253 under these conditions the additional ligand binding site of the dimeric bOBP/OCT complex is
254 occupied by ANS, whereas the inner cavity of the barrel is engaged in the ligand binding. It is
255 likely that the inability of the natural ligand to interact with the additional weak ligand binding
256 site located between the monomeric subunits can be due to the structural difference of this site in
257 the native dimeric bOBP and in a protein in the original native-like state or an intermediate
258 compact state.

259 Analysis of the bOBP/OCT refolding from the completely unfolded state revealed that the
260 dependencies of various structural characteristics of the bOBP/OCT on GdnHCl concentrations
261 depend on the incubation time of this complex in the presence of the denaturant (see Figure 4). In
262 fact, during the refolding process, equilibrium values of the analyzed structural characteristics of
263 the bOBP/OCT complex are reached after the incubation of this complex in the presence of the
264 desired GdnHCl concentration for 72 hrs. No subsequent changes were detected when protein
265 was incubated for 30 days. This analysis revealed the presence of noticeable hysteresis between
266 the curves describing the equilibrium unfolding and refolding of the bOBP/OCT complex in a
267 wide range of the GdnHCl concentrations. In fact, the equilibrium unfolding and refolding
268 curves coincide only in the vicinity of 2.0 M GdnHCl, where, according to the gel-filtration

269 analysis, the native dimeric state of the bOBP/OCT complex is formed, whereas within the
270 region corresponding to the transition from the native dimeric form to the completely unfolded
271 state of the bOBP/OCT complex, equilibrium curves describing unfolding and refolding of this
272 complex do not coincide.

273 The equilibrium refolding curve describing transition from the unfolded to the compact
274 dimeric state of the bOBP/OCT complex is shifted toward the lower GdnHCl concentrations in
275 comparison with the equilibrium unfolding curve (Figure 4). However, in comparison with the
276 unfolding of the bOBP alone, this equilibrium refolding curve of the bOBP/OCT complex is still
277 shifted toward higher GdnHCl concentrations. These data suggest that at the same denaturant
278 concentrations, the fractions of native bOBP formed during the refolding from the completely
279 unfolded state are significantly lower than the fraction of native protein remaining within the
280 region of the bOBP/OCT unfolding. However, once formed, the native protein gains the ability
281 to bind ligand. This hypothesis is supported by the results of the gel-filtration analysis (Figure 3).
282 For example, the elution profiles registered during the unfolding and refolding of the bOBP/OCT
283 complex at 2.5 M GdnHCl show that under these conditions, more native protein is present
284 during the bOBP/OCT unfolding, whereas unfolded species prevail during the refolding of this
285 complex. Therefore, the effective rates of the formation of various bOBP conformers are
286 significantly different during the unfolding and refolding processes and noticeably depend on the
287 denaturant concentration.

288 It is likely that the same reasons define the irreversibility of the unfolding of the
289 bOBP/OCT complex at low GdnHCl concentrations. Under these conditions, the rate of the
290 formation of the monomeric bOBP/OCT complex is significantly higher than the rate of the
291 dimeric bOBP/OCT formation. As a result, refolding of the bOBP/OCT complex at the low

292 GdnHCl concentrations results in the preferential formation of monomeric bOBP/OCT species,
293 whereas under the identical conditions, the unfolding reaction mixture contains roughly
294 equimolar quantities of the bOBP/OCT monomers and dimmers (Figures 3 and 4).

295

296 *Equilibrium unfolding of the monomeric bOBP-Gly121+*

297 Already at relatively low GdnHCl concentrations, the monomeric bOBP-Gly121+ is
298 converted to the compact partially folded state with structural characteristics resembling those of
299 the partially folded species accumulated during the equilibrium unfolding of the recombinant
300 bOBP (see Figures 5-6 and Table 2). This compact intermediate is able to bind ANS and exists in
301 a wide range of the GdnHCl concentrations (up to about 1.3 M GdnHCl). Subsequent increase in
302 the denaturant concentration promotes transition to a more loose form, which, at the further
303 increase of the GdnHCl concentration, is converted to the completely unfolded state. This
304 GdnHCl-induced unfolding of the bOBP-Gly121+ is a completely reversible process as
305 evidenced by the coincidence of the equilibrium characteristics of the protein measured at the
306 processes of the bOBP-Gly121+ unfolding and refolding.

307 The formation of the bOBP-Gly121+/OCT complex results in a noticeable stabilization of
308 this protein. This is evidenced by the increase in the cooperativity of the unfolding transition,
309 which is also shifted toward higher GdnHCl concentrations. However, the formation of a
310 complex between the bOBP-Gly121+ and OCT does not affect the unfolding mechanism of this
311 protein.

312

313 *GdnHCl-induced unfolding of the monomeric GCC-bOBP*

314 Analysis of the peculiarities of the equilibrium unfolding and refolding processes
315 monitored by the GdnHCl-induced changes in various structural characteristics of the
316 monomeric GCC-bOBP suggests that the unfolding of this protein is a completely reversible
317 process accompanied by the formation of partially folded intermediates similar to those observed
318 during the equilibrium unfolding of the recombinant bOBP and its monomeric bOBP-Gly121+
319 form (see Figures 7-8 and Table 2). However, although qualitatively unfolded processes of these
320 three proteins are similar, there are some noticeable differences. For example, in comparison
321 with the recombinant bOBP and bOBP-Gly121+ unfolding, a compact intermediate with high
322 ANS affinity is formed at higher denaturant concentrations during the GCC-bOBP unfolding (at
323 1.0 M GdnHCl). This illustrates higher conformational stability of the disulfide-stabilized GCC-
324 bOBP compared to the recombinant bOBP and its monomeric form bOBP-Gly121+.

325 GCC-bOBP is further stabilized due to the GCC-bOBP/OCT complex formation.
326 Refolding curves detected by changes in different structural characteristics of this complex and
327 registered after the incubation of the corresponding solutions for one hour coincide with the
328 transition curves describing the equilibrium unfolding of GCC-bOBP, and subsequent incubation
329 of these same solutions for 72 hrs leads to the detectable shift of the transition curves. As a
330 result, equilibrium unfolding and refolding transitions of the GCC-bOBP/OCT complex coincide
331 suggesting that the unfolding of this protein is a completely reversible process. However, GCC-
332 bOBP becomes able to bind ligand only after the formation of correct native structure stabilized
333 by the disulfide bond. Earlier similar effects were described for other ligand-binding protein,
334 such as the D-glucose/D-galactose-binding protein (GGBP) from *E. coli* (Stepanenko et al.
335 2011a; Stepanenko et al. 2009; Stepanenko et al. 2011b). In fact, our analysis of the peculiarities
336 of the GGBP unfolding revealed that ligand binding might constitute a rate-limiting stage of the

337 protein unfolding-refolding process. This phenomenon can be understood considering the fact
338 that the formation of the protein-ligand complex depends on the appearance of the matching
339 configurations between the ligand and the active site of a fully formed native protein
340 (Stepanenko et al. 2011a; Stepanenko et al. 2009; Stepanenko et al. 2011b).

341 Our current analysis revealed that the unfolding of the monomeric complexes bOBP-
342 Gly121+/OCT and GCC-bOBP/OCT is not accompanied by the ANS fluorescence enhancement
343 in the whole range of GdnHCl concentrations (see Figures 6 and 8). These observations support
344 the hypothesis on the existence of the additional ligand-binding site in the dimeric bOBP.

345 Figure 9 shows that the equilibrium unfolding transition recorded for the recombinant
346 bOBP coincides with that of its monomeric bOBP-Gly121+ form. On the other hand, unfolding
347 transition of the monomeric GCC-bOBP stabilized by the engineered disulfide bond is noticeably
348 shifted to higher denaturant concentrations. Curiously, the unfolding of complexes of all proteins
349 analyzed in this study with natural ligand OCT happens at the same denaturant concentrations.

350 Our data suggest that protein dimerization via the domain-swapping mechanism does not
351 contribute much to the increase in the conformational stability of a protein (at least in the case of
352 the analyzed in this study bOBP), despite the fact that the increased conformational stability was
353 proposed as one of the factors determining the use of this mechanism for dimer and higher
354 oligomer formation (Bennett et al. 1995; Liu & Eisenberg 2002). In contrast, introduction of a
355 disulfide bond to the structure of a monomeric protein shows significant stabilizing effects. Our
356 data also show that the formation of a protein-ligand complex leads to the significant
357 stabilization of different variants of bOBP and eliminates the original difference in
358 conformational stability caused by their structural differences.

359

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363

364 **AUTHOR CONTRIBUTIONS**

365 Olga VS, Olesya VS and DOR collected and analyzed data, contributed to discussion, and wrote
366 the manuscript. IMK and KKT conceived the idea, supervised the project, contributed to
367 discussion, and reviewed/edited manuscript. VNU analyzed data, contributed to discussion, and
368 wrote the manuscript.

369

370 **DISCLOSURE**

371 None declared.

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507 **FIGURE LEGENDS**

508

509 **Figure 1. Analysis of the 3D structure of bOBP.** Crystal 3D structures of natural bOBP (**A**)
510 and monomeric mutant form GCC-bOBP (**B**). The individual subunits in the bOBP are in gray
511 and orange. In the GCC-bOBP short α -helical segment that followed by the 9th β -strand and the
512 disordered C-terminal region of the protein are drawn in orange. The tryptophan residues are
513 indicated in red and blue in the different subunits of bOBP and in blue in GCC-bOBP. The Gly
514 121+ residue which donates the increased mobility of the loop connecting α -helix and 8th β -
515 strand of the β -barrel and promotes the formation of a monomeric fold of the mutant protein
516 bOBP-Gly121+ is in green. Two cysteines residues Cys 64 and Cys 156 in GCC-bOBP, which
517 are believed to stabilize monomeric structure due to the disulfide bond formation are in yellow.
518 The drawing of bOBP and GCC-bOBP was generated based on the 1OBP (Tegoni et al. 1996)
519 and 2HLV files (Ramoni et al. 2008) from PDB (Dutta et al. 2009) using the graphic software
520 VMD (Hsin et al. 2008) and Raster3D (Merritt & Bacon 1977). Plot **C** represents the results of
521 the multiple structural alignment of bOBP (PDB ID: 1OBP, blue structure), GCC-bOBP (PDB
522 ID: 2HLV, red structure), and naturally monomeric pOBP (PDB ID: 1A3Y, green structure)
523 using the MultiProt algorithm (<http://bioinfo3d.cs.tau.ac.il/MultiProt/>) (Shatsky et al. 2004). The
524 drawing was generated using the graphic software VMD (Hsin et al. 2008). Plot **D** compares
525 flexibility profiles obtained from crystal structures of bOBP (PDB ID: 1OBP, black and red lines
526 for the chains A and B), naturally monomeric pOBP (PDB ID: 1A3Y, green and yellow lines for
527 the chains A and B) and monomeric mutant GCC-bOBP (PDB ID: 2HLV, blue line) with the
528 intrinsic disorder propensity of the bOBP (UniProt ID: P07435, pink dashed line). Flexibility
529 profiles were obtained using the FlexPred software available at <http://kiharalab.org/flexPred/>
530 (Jamroz et al. 2012), whereas intrinsic disorder propensity was evaluated using the PONDR®
531 VSL2 algorithm (Peng et al. 2005).

532

533 **Figure 2. bOBP conformational changes induced by GdnHCl (the data are from**
534 **(Stepanenko et al. 2014c)).** **A:** changes in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; **B:**
535 changes in parameter A , $\lambda_{\text{ex}}=297$ nm; **C:** changes in fluorescence anisotropy at the emission
536 wavelength 365 nm, $\lambda_{\text{ex}}=297$ nm; **D:** changes in the ellipticity at 222 nm. The measurements
537 were preceded by incubating the protein in a solution with the appropriate GdnHCl concentration
538 at 4 °C for 24 h. The open symbols indicate unfolding, whereas the closed symbols represent
539 refolding. Changes in bOBP hydrodynamic dimensions for the different structural states were
540 followed by the changes in the elution profiles for bOBP after pre-incubation for 24 h (solid
541 lines) and 43 h (dashed line) with GdnHCl at the concentrations 0.0 (**E**), 0.5 (**F**) and 1.5 (**G**) for
542 the denaturation process.

543

544 **Figure 3. The changes of hydrodynamic dimensions of recombinant bOBP (A and B) and**
545 **its complex with ligand bOBP/OCT (C and D) in different structural states.** The elution
546 profiles for bOBP and bOBP/OCT were recorded during the protein denaturation (**A** and **C**) and
547 renaturation from unfolded states (**B** and **D**) induced by GdnHCl. The elution profiles for bOBP
548 were measured after pre-incubation of the protein and the solution of GdnHCl in desired
549 concentration for 24h (**A** and **B**), while in the case of bOBP/OCT the incubation time was
550 extended to 72 – 84 h for denaturation (**C**) and 6 days for renaturation (**D**). The figures on the
551 curves are the GdnHCl concentrations.

552

553 **Figure 4. bOBP and bOBP/OCT conformational changes induced by GdnHCl.** *A*: changes
554 in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; *B*: changes in parameter *A*, $\lambda_{\text{ex}}=297$ nm; *C*:
555 changes in fluorescence anisotropy at the emission wavelength 365 nm, $\lambda_{\text{ex}}=297$ nm; *D*: changes
556 in the ellipticity at 222 nm; *E*: changes in the ANS fluorescence intensity at $\lambda_{\text{ex}}=365$ nm,
557 $\lambda_{\text{em}}=480$ nm.

558 The measurements were preceded by incubating the protein in a solution with the appropriate
559 GdnHCl concentration at 4 °C for 24 (red circles) in the case of bOBP. The open symbols
560 indicate unfolding, whereas the closed symbols represent refolding.

561 While studying the folding of bOBP/OCT (squares), the solution of complex of the protein with
562 its ligand were incubated in a solution with the appropriate GdnHCl concentration at 4 °C for less
563 than 24 h (open brown squares), up to 120 h (open pink squares) at the protein denaturation, and
564 1 h (closed brown squares) and 72 h – 30 days (closed pink squares) at the protein renaturation.

565

566 **Figure 5. The changes of hydrodynamic dimensions of recombinant bOBP-Gly121+ (*A* and
567 *B*) and its complex with ligand bOBP-Gly121+/OCT (*C* and *D*) in different structural
568 states.** The elution profiles for bOBP-Gly121+ and bOBP-Gly121+/OCT were recorded during
569 the protein denaturation (*A* and *C*) and renaturation from unfolded states (*B* and *D*) induced by
570 GdnHCl. The elution profiles for bOBP-Gly121+ were measured after pre-incubation of the
571 protein and the solution of GdnHCl in desired concentration for 24h (*A* and *B*), while in the case
572 of bOBP-Gly121+/OCT the incubation time was extended to 72 – 84 h for denaturation (*C*) and
573 6 days for renaturation (*D*). The figures on the curves are the GdnHCl concentrations.

574

575 **Figure 6. Figure 4. bOBP-Gly121+ and bOBP-Gly121+/OCT conformational changes
576 induced by GdnHCl.** *A*: changes in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; *B*: changes
577 in parameter *A*, $\lambda_{\text{ex}}=297$ nm; *C*: changes in fluorescence anisotropy at the emission wavelength
578 365 nm, $\lambda_{\text{ex}}=297$ nm; *D*: changes in the ellipticity at 222 nm; *E*: changes in the ANS
579 fluorescence intensity at $\lambda_{\text{ex}}=365$ nm, $\lambda_{\text{em}}=480$ nm.

580 The measurements were preceded by incubating the protein in a solution with the appropriate
581 GdnHCl concentration at 4 °C for 24 (dark green circles) in the case of bOBP-Gly121+. The
582 open symbols indicate unfolding, whereas the closed symbols represent refolding.

583 While studying the folding of bOBP-Gly121+/OCT (squares), the solution of complex of the
584 protein with its ligand were incubated in a solution with the appropriate GdnHCl concentration at
585 4 °C for less than 24 h (open dark yellow squares), up to 72 h (open light green squares) at the
586 protein denaturation, and 1 h (closed dark yellow squares) and 72 h (closed light green squares)
587 at the protein renaturation.

588

589

590 **Figure 7. The changes of hydrodynamic dimensions of recombinant GCC-bOBP (*A* and *B*)
591 and its complex with ligand GCC-bOBP/OCT (*C* and *D*) in different structural states.** The
592 elution profiles for GCC-bOBP and GCC-bOBP/OCT were recorded during the protein
593 denaturation (*A* and *C*) and renaturation from unfolded states (*B* and *D*) induced by GdnHCl.
594 The elution profiles for GCC-bOBP were measured after pre-incubation of the protein and the
595 solution of GdnHCl in desired concentration for 24h (*A* and *B*), while in the case of GCC-
596 bOBP/OCT the incubation time was extended to 72 – 84 h for denaturation (*C*) and 6 days for
597 renaturation (*D*). The figures on the curves are the GdnHCl concentrations.

598

599 **Figure 8. GCC-bOBP and GCC-bOBP/OCT conformational changes induced by GdnHCl.**

600 **A:** changes in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; **B:** changes in parameter A , $\lambda_{\text{ex}}=297$
601 nm; **C:** changes in fluorescence anisotropy at the emission wavelength 365 nm, $\lambda_{\text{ex}}=297$ nm; **D:**
602 changes in the ellipticity at 222 nm; **E:** changes in the ANS fluorescence intensity at $\lambda_{\text{ex}}=365$ nm,
603 $\lambda_{\text{em}}=480$ nm.

604 The measurements were preceded by incubating the protein in a solution with the appropriate
605 GdnHCl concentration at 4 °C for 24 (blue circles) in the case of GCC-bOBP. The open symbols
606 indicate unfolding, whereas the closed symbols represent refolding.

607 While studying the folding of GCC-bOBP/OCT (squares), the solution of complex of the protein
608 with its ligand were incubated in a solution with the appropriate GdnHCl concentration at 4 °C
609 for less than 24 h (open dark blue squares), up to 72 h (open light blue squares) at the protein
610 denaturation, and 1 h (closed dark blue squares) and 72 h (closed light blue squares) at the
611 protein renaturation.

612

613 **Figure 9. Conformational changes of bOBP (red circles), bOBP-Gly121+ (green circles)**
614 **and GCC-bOBP (blue circles) and their complexes bOBP/OCT (pink squares), bOBP-**
615 **Gly121+/OCT (light green squares) and GCC-bOBP (light blue squares) induced by**
616 **GdnHCl. A:** changes in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; **B:** changes in parameter
617 A , $\lambda_{\text{ex}}=297$ nm; **C:** changes in fluorescence anisotropy at the emission wavelength 365 nm,
618 $\lambda_{\text{ex}}=297$ nm; **D:** changes in the ellipticity at 222 nm.

619 The equilibrium data for all studied proteins are compared. The open symbols indicate unfolding,
620 whereas the closed symbols represent refolding.

Table 1 (on next page)

These are tables 1 and 2

1 **Table 1.** Characteristics of intrinsic fluorescence of recombinant bOBP in different structural
2 states

Parameter	bOBP in buffered solution	bOBP in state I ₁ (0.5 M GdnHCl)	bOBP in state I ₂ (1.6 M GdnHCl)
λ_{\max} , nm ($\lambda_{\text{ex}}=297$ nm)	335	337	335
Parameter <i>A</i> ($\lambda_{\text{ex}}=297$ nm)	1.2	1.1	1.2
<i>r</i> ($\lambda_{\text{ex}}=297$ nm, $\lambda_{\text{em}}=365$ nm)	0.170	0.166	0.180
τ , ns ($\lambda_{\text{ex}}=297$ nm, $\lambda_{\text{em}}=335$ nm)	4.4± 0.2	4.6±0.2	4.8±0.1

3
4 The data are from (Stepanenko et al. 2014c).

5
6
7 **Table 2.** Hydrodynamic dimensions of recombinant bOBPwt and its mutant forms in the absence
8 and in the presence of natural ligand OCT in different structural states.

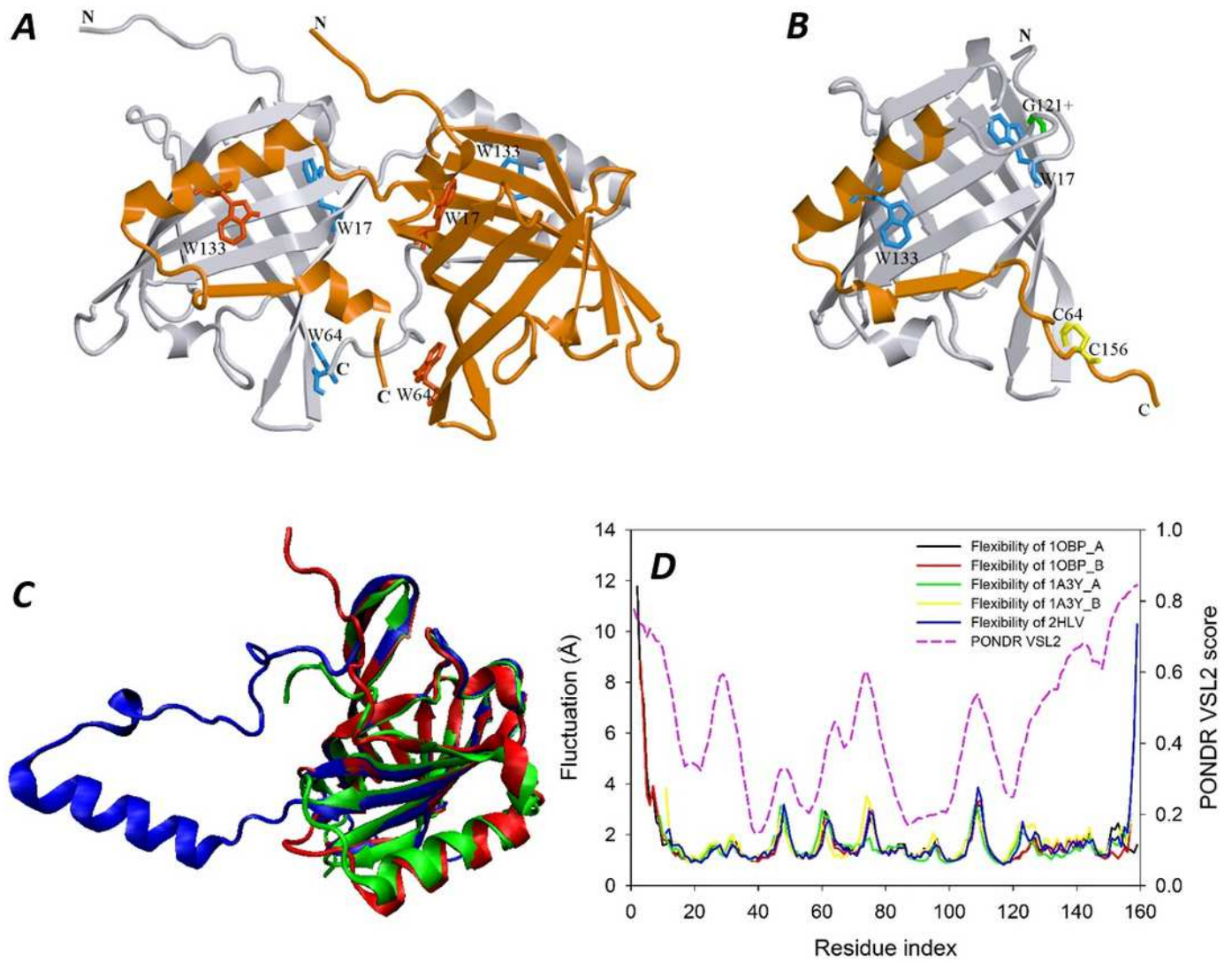
	GdnHCl, M	1 peak, kDa	2 peak, kDa
bOBPwt	0.0	43.9	23.8
	0.5	34.0	19.3
	1.5	43.6	
bOBPwt/OCT	0.0	39.6	21.5
	0.55	27.2	17.0
	1.7	39.6	
bOBP/Gly121+	0.0	23.6	
	0.25	17.8	
	1.5 – 1.9	24.8 – 28.5	
bOBP/Gly121+/OCT	0.0	21.5	
	0.24 – 0.5	15.5 – 16.2	
	1.7 – 2.0	24.7 – 28.5	
GCC-bOBP	0.0	23.6	
	0.25	17.8	
	1.1 – 1.82	22.5 – 25.9	
GCC-bOBP/OCT	0.0	22.5	
	0.27	16.9	
	1.5 – 2.0	22.5 – 27.2	

9
10

1

Analysis of the 3D structure of bOBP.

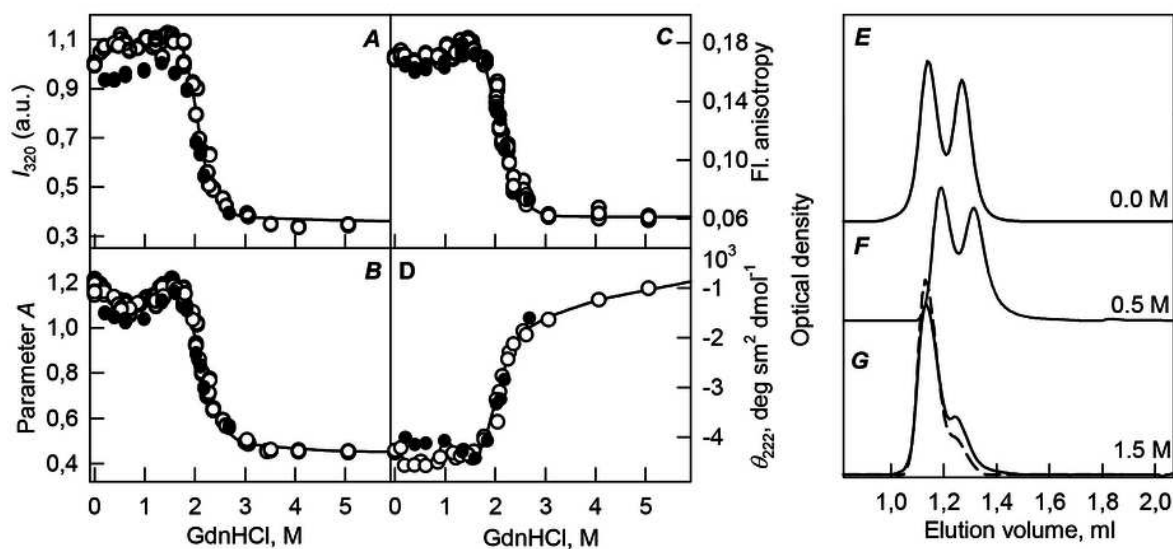
Crystal 3D structures of natural bOBP (**A**) and monomeric mutant form GCC-bOBP (**B**). The individual subunits in the bOBP are in gray and orange. In the GCC-bOBP short α -helical segment that followed by the 9th β -strand and the disordered C-terminal region of the protein are drawn in orange. The tryptophan residues are indicated in red and blue in the different subunits of bOBP and in blue in GCC-bOBP. The Gly 121+ residue which donates the increased mobility of the loop connecting α -helix and 8th β -strand of the β -barrel and promotes the formation of a monomeric fold of the mutant protein bOBP-Gly121+ is in green. Two cysteines residues Cys 64 and Cys 156 in GCC-bOBP, which are believed to stabilize monomeric structure due to the disulfide bond formation are in yellow. The drawing of bOBP and GCC-bOBP was generated based on the 1OBP (Tegoni et al. 1996) and 2HLV files (Ramoni et al. 2008) from PDB (Dutta et al. 2009) using the graphic software VMD (Hsin et al. 2008) and Raster3D (Merritt & Bacon 1977). Plot **C** represents the results of the multiple structural alignment of bOBP (PDB ID: 1OBP, blue structure), GCC-bOBP (PDB ID: 2HLV, red structure), and naturally monomeric pOBP (PDB ID: 1A3Y, green structure) using the MultiProt algorithm (<http://bioinfo3d.cs.tau.ac.il/MultiProt/>) (Shatsky et al. 2004). The drawing was generated using the graphic software VMD (Hsin et al. 2008). Plot **D** compares flexibility profiles obtained from crystal structures of bOBP (PDB ID: 1OBP, black and red lines for the chains A and B), naturally monomeric pOBP (PDB ID: 1A3Y, green and yellow lines for the chains A and B) and monomeric mutant GCC-bOBP (PDB ID: 2HLV, blue line) with the intrinsic disorder propensity of the bOBP (UniProt ID: P07435, pink dashed line). Flexibility profiles were obtained using the FlexPred software available at <http://kiharalab.org/flexPred/> (Jamroz et al. 2012), whereas intrinsic disorder propensity was evaluated using the PONDR® VSL2 algorithm (Peng et al. 2005).



2

bOBP conformational changes induced by GdnHCl (the data are from (Stepanenko et al. 2014c)).

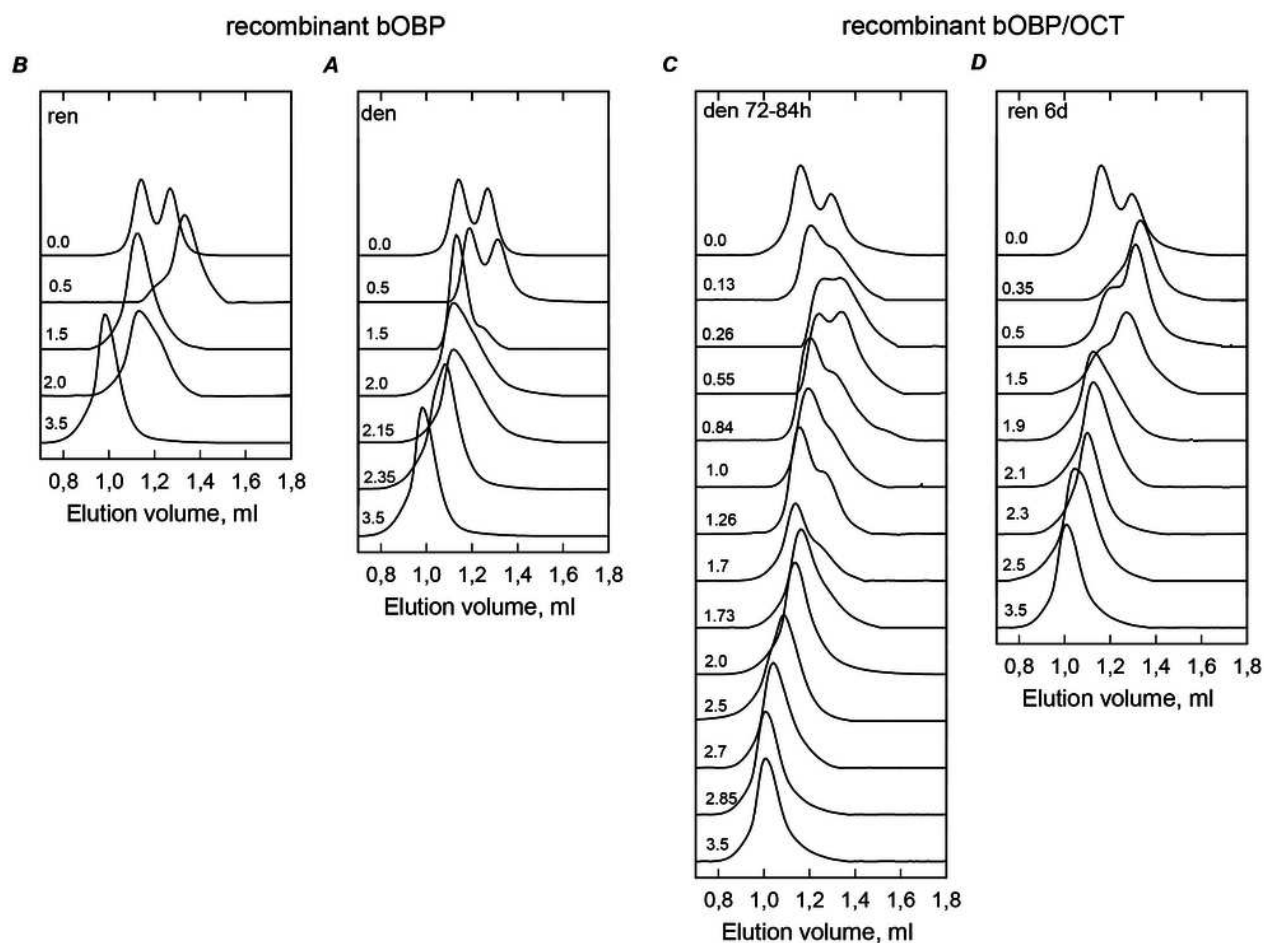
A: changes in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; **B**: changes in parameter A, $\lambda_{\text{ex}}=297$ nm; **C**: changes in fluorescence anisotropy at the emission wavelength 365 nm, $\lambda_{\text{ex}}=297$ nm; **D**: changes in the ellipticity at 222 nm. The measurements were preceded by incubating the protein in a solution with the appropriate GdnHCl concentration at 4 °C for 24 h. The open symbols indicate unfolding, whereas the closed symbols represent refolding. Changes in bOBP hydrodynamic dimensions for the different structural states were followed by the changes in the elution profiles for bOBP after pre-incubation for 24 h (solid lines) and 43 h (dashed line) with GdnHCl at the concentrations 0.0 (**E**), 0.5 (**F**) and 1.5 (**G**) for the denaturation process.



3

The changes of hydrodynamic dimensions of recombinant bOBP (**A** and **B**) and its complex with ligand bOBP/OCT (**C** and **D**) in different structural states.

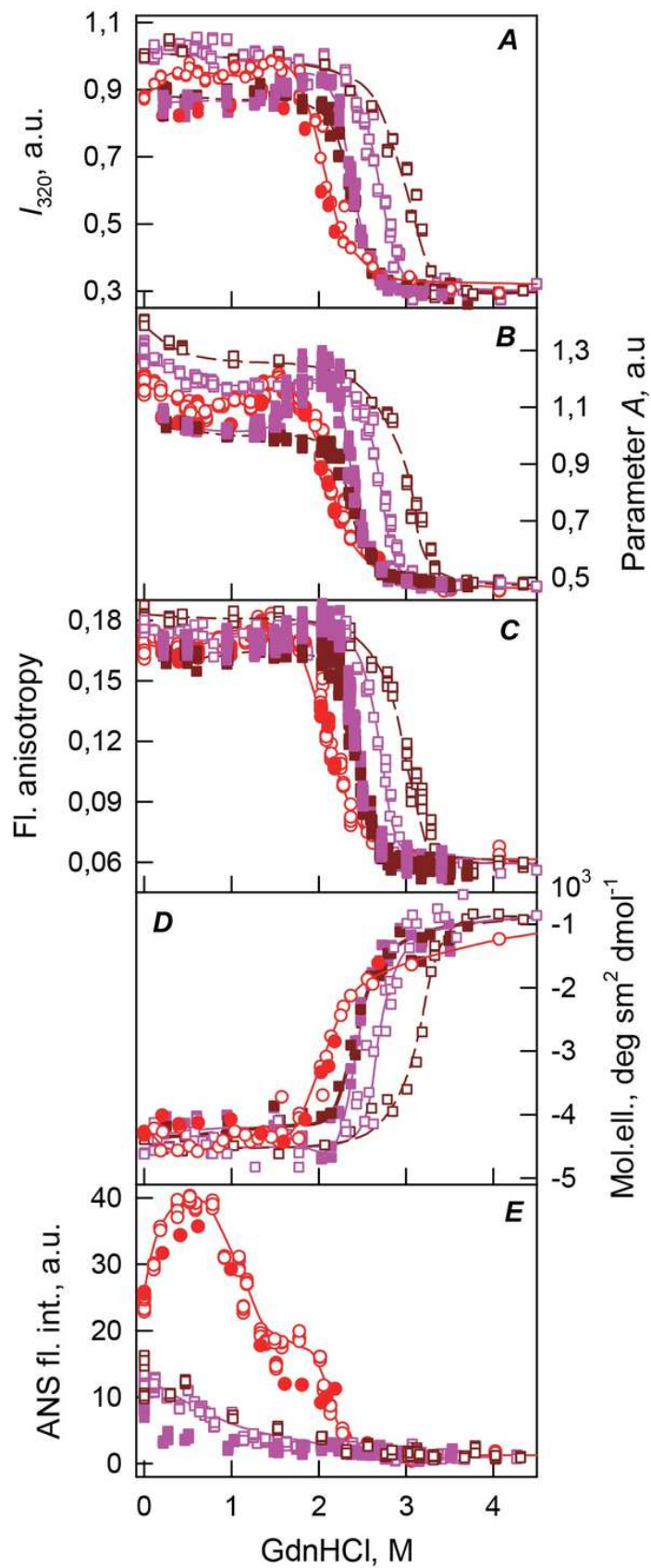
The elution profiles for bOBP and bOBP/OCT were recorded during the protein denaturation (**A** and **C**) and renaturation from unfolded states (**B** and **D**) induced by GdnHCl. The elution profiles for bOBP were measured after pre-incubation of the protein and the solution of GdnHCl in desired concentration for 24h (**A** and **B**), while in the case of bOBP/OCT the incubation time was extended to 72 - 84 h for denaturation (**C**) and 6 days for renaturation (**D**). The figures on the curves are the GdnHCl concentrations.



4

bOBP and bOBP/OCT conformational changes induced by GdnHCl.

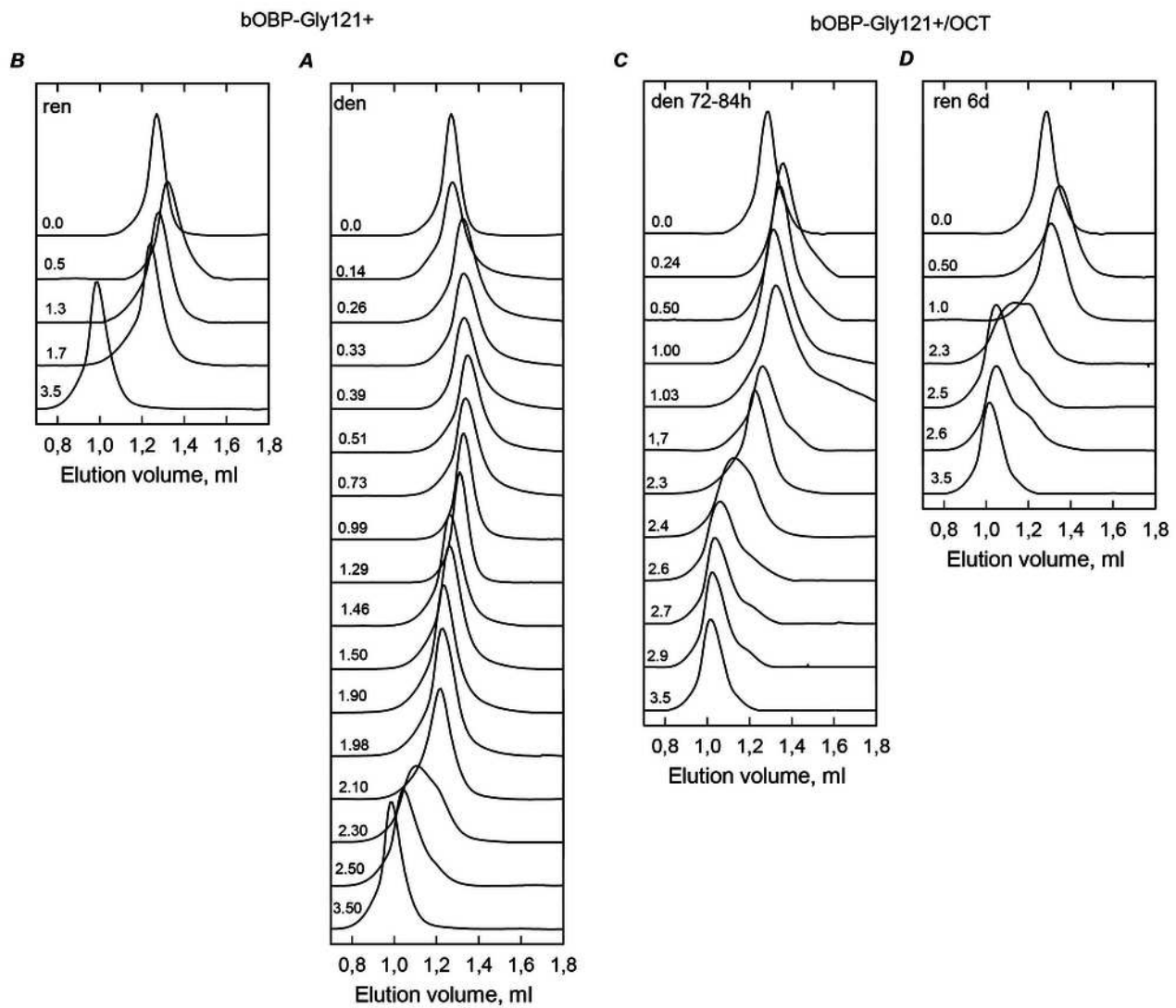
A: changes in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; **B**: changes in parameter A , $\lambda_{\text{ex}}=297$ nm; **C**: changes in fluorescence anisotropy at the emission wavelength 365 nm, $\lambda_{\text{ex}}=297$ nm; **D**: changes in the ellipticity at 222 nm; **E**: changes in the ANS fluorescence intensity at $\lambda_{\text{ex}}=365$ nm, $\lambda_{\text{em}}=480$ nm. The measurements were preceded by incubating the protein in a solution with the appropriate GdnHCl concentration at 4 °C for 24 (red circles) in the case of bOBP. The open symbols indicate unfolding, whereas the closed symbols represent refolding. While studying the folding of bOBP/OCT (squares), the solution of complex of the protein with its ligand were incubated in a solution with the appropriate GdnHCl concentration at 4 °C for less than 24 h (open brown squares), up to 120 h (open pink squares) at the protein denaturation, and 1 h (closed brown squares) and 72 h - 30 days (closed pink squares) at the protein renaturation.



5

The changes of hydrodynamic dimensions of recombinant bOBP-Gly121+ (*A* and *B*) and its complex with ligand bOBP-Gly121+/OCT (*C* and *D*) in different structural states.

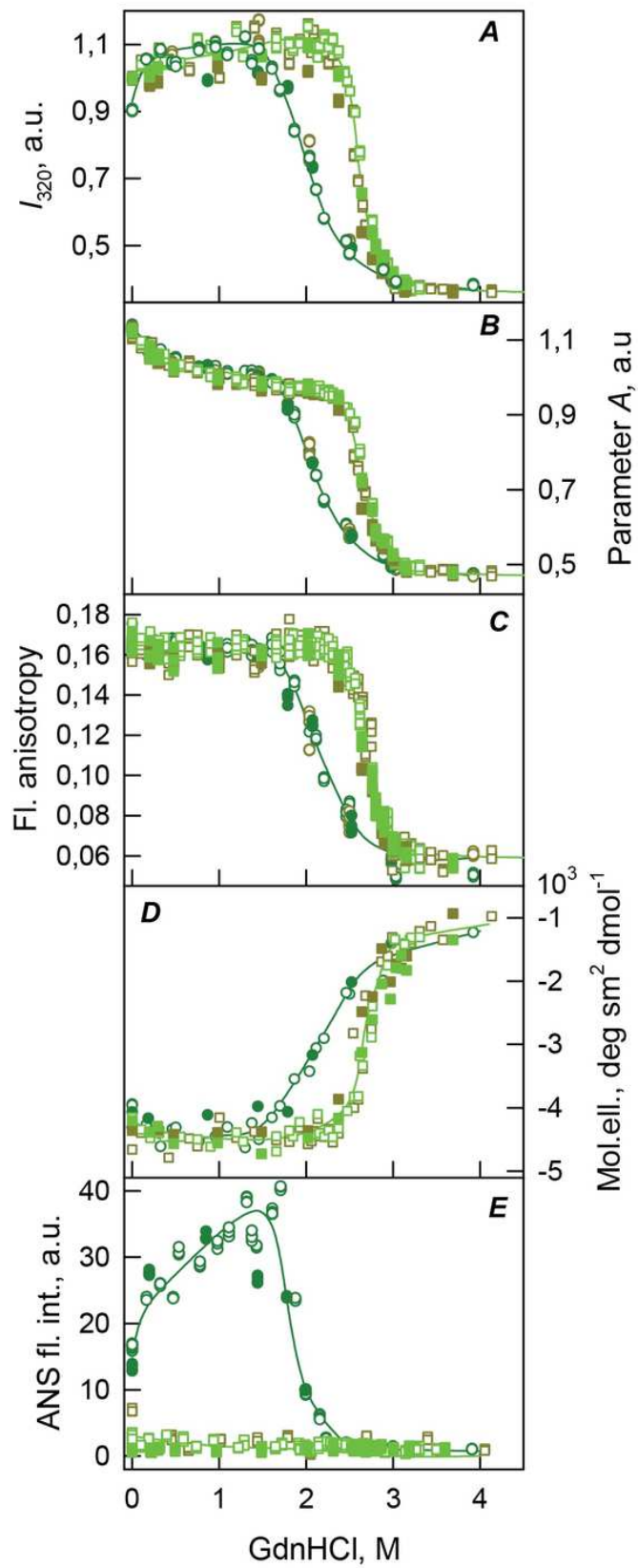
The elution profiles for bOBP-Gly121+ and bOBP-Gly121+/OCT were recorded during the protein denaturation (**A** and **C**) and renaturation from unfolded states (**B** and **D**) induced by GdnHCl. The elution profiles for bOBP-Gly121+ were measured after pre-incubation of the protein and the solution of GdnHCl in desired concentration for 24h (**A** and **B**), while in the case of bOBP-Gly121+/OCT the incubation time was extended to 72 - 84 h for denaturation (**C**) and 6 days for renaturation (**D**). The figures on the curves are the GdnHCl concentrations.



6

Figure 4. bOBP-Gly121+ and bOBP-Gly121+/OCT conformational changes induced by GdnHCl.

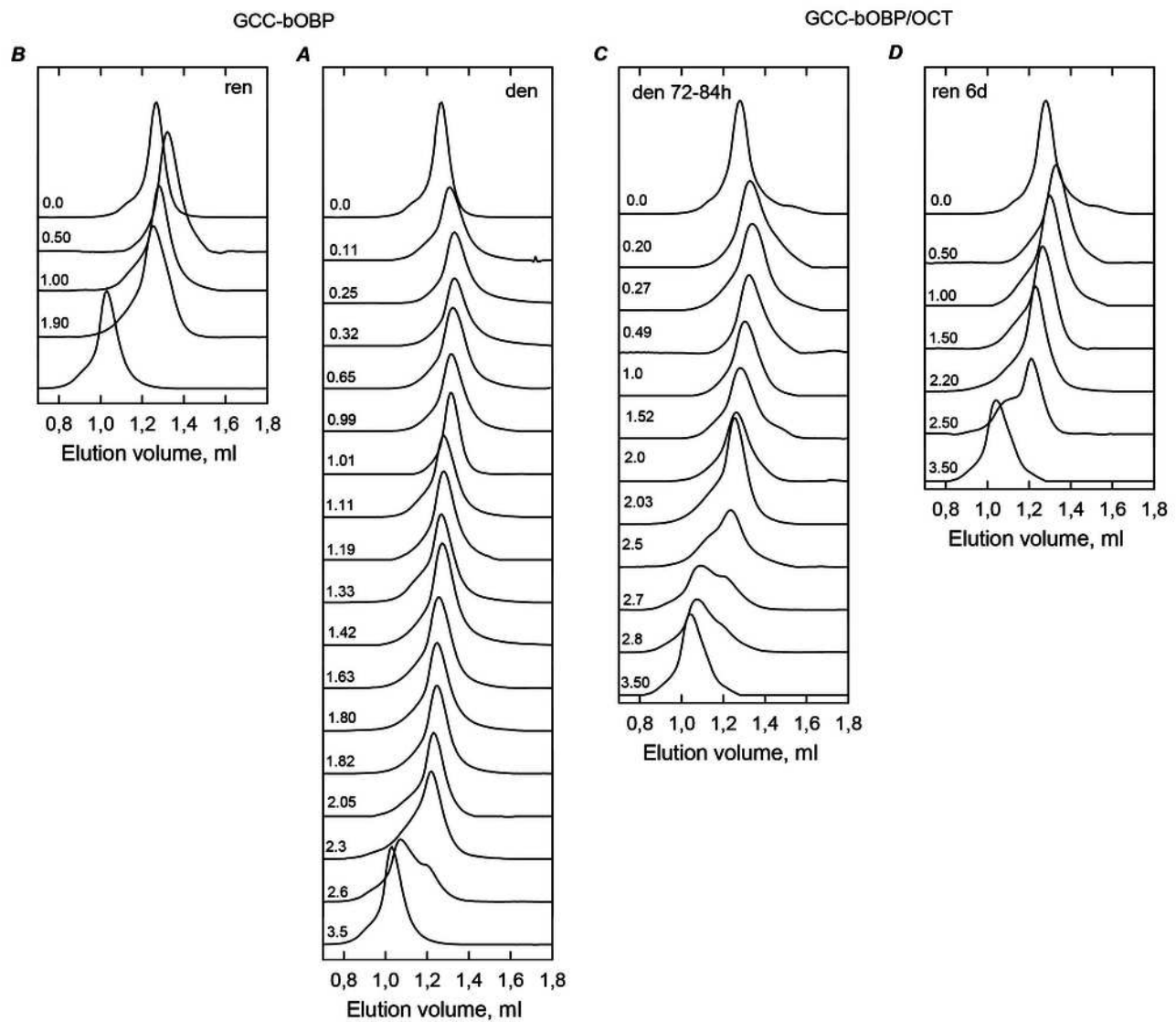
A: changes in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; **B**: changes in parameter A, $\lambda_{\text{ex}}=297$ nm; **C**: changes in fluorescence anisotropy at the emission wavelength 365 nm, $\lambda_{\text{ex}}=297$ nm; **D**: changes in the ellipticity at 222 nm; **E**: changes in the ANS fluorescence intensity at $\lambda_{\text{ex}}=365$ nm, $\lambda_{\text{em}}=480$ nm. The measurements were preceded by incubating the protein in a solution with the appropriate GdnHCl concentration at 4 °C for 24 h (dark green circles) in the case of bOBP-Gly121+. The open symbols indicate unfolding, whereas the closed symbols represent refolding. While studying the folding of bOBP-Gly121+/OCT (squares), the solution of complex of the protein with its ligand were incubated in a solution with the appropriate GdnHCl concentration at 4 °C for less than 24 h (open dark yellow squares), up to 72 h (open light green squares) at the protein denaturation, and 1 h (closed dark yellow squares) and 72 h (closed light green squares) at the protein renaturation.



7

The changes of hydrodynamic dimensions of recombinant GCC-bOBP (*A* and *B*) and its complex with ligand GCC-bOBP/OCT (*C* and *D*) in different structural states.

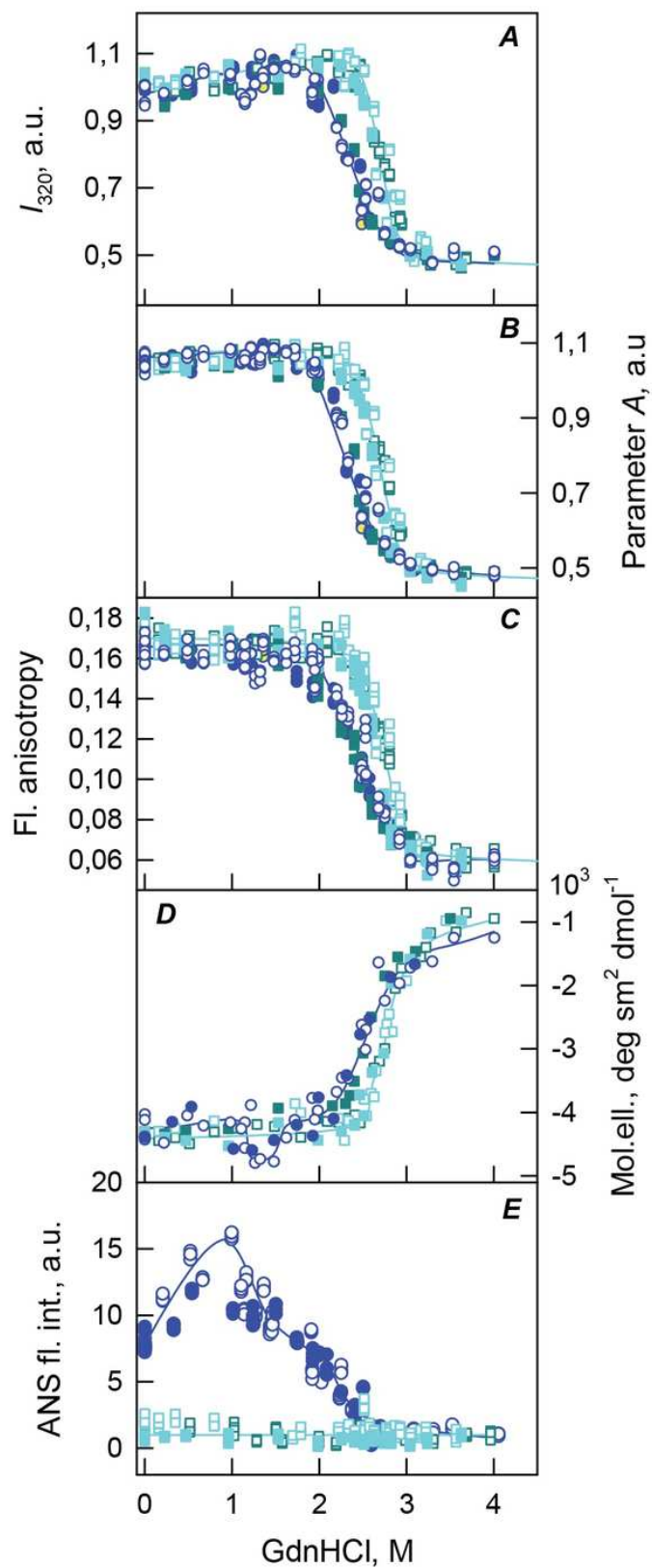
The elution profiles for GCC-bOBP and GCC-bOBP/OCT were recorded during the protein denaturation (**A** and **C**) and renaturation from unfolded states (**B** and **D**) induced by GdnHCl. The elution profiles for GCC-bOBP were measured after pre-incubation of the protein and the solution of GdnHCl in desired concentration for 24h (**A** and **B**), while in the case of GCC-bOBP/OCT the incubation time was extended to 72 - 84 h for denaturation (**C**) and 6 days for renaturation (**D**). The figures on the curves are the GdnHCl concentrations.



8

GCC-bOBP and GCC-bOBP/OCT conformational changes induced by GdnHCl.

A: changes in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; **B**: changes in parameter A , $\lambda_{\text{ex}}=297$ nm; **C**: changes in fluorescence anisotropy at the emission wavelength 365 nm, $\lambda_{\text{ex}}=297$ nm; **D**: changes in the ellipticity at 222 nm; **E**: changes in the ANS fluorescence intensity at $\lambda_{\text{ex}}=365$ nm, $\lambda_{\text{em}}=480$ nm. The measurements were preceded by incubating the protein in a solution with the appropriate GdnHCl concentration at 4 °C for 24 (blue circles) in the case of GCC-bOBP. The open symbols indicate unfolding, whereas the closed symbols represent refolding. While studying the folding of GCC-bOBP/OCT (squares), the solution of complex of the protein with its ligand were incubated in a solution with the appropriate GdnHCl concentration at 4 °C for less than 24 h (open dark blue squares), up to 72 h (open light blue squares) at the protein denaturation, and 1 h (closed dark blue squares) and 72 h (closed light blue squares) at the protein renaturation.



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Figure 9. Conformational changes of bOBP (red circles), bOBP-Gly121+ (green circles) and GCC-bOBP (blue circles) and their complexes bOBP/OCT (pink squares), bOBP-Gly121+/OCT (light green squares) and GCC-bOBP (light blue squares) induced by GdnHCl.

A: changes in fluorescence intensity at 320 nm, $\lambda_{\text{ex}}=297$ nm; **B**: changes in parameter A, $\lambda_{\text{ex}}=297$ nm; **C**: changes in fluorescence anisotropy at the emission wavelength 365 nm, $\lambda_{\text{ex}}=297$ nm; **D**: changes in the ellipticity at 222 nm. The equilibrium data for all studied proteins are compared. The open symbols indicate unfolding, whereas the closed symbols represent refolding.

