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DENATURATION OF PHYCOCYANIN BY UREA AND DETERMINATION OF THE ENTHALPY OF DENATURATION BY MICROCALORIMETRY

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Denaturation of the protein phycocyanin in urea solution was investigated by microcalorimetry, ultraviolet and visible spectroscopy, circular dichroism and sedimentation equilibrium. The results consistently demonstrated that in the presence of 7 M urea this protein is completely denatured. By assuming a two-state mechanism, an apparent free energy of unfolding at zero denaturant concentration, $\Delta G^{H_2O}_{app}$, was found to be 4.4 kcal/mole at pH 6.0 and 25°C. By microcalorimetry the enthalpy of denaturation of phycocyanin was found to be −230 kcal/mole at 25°C. The relatively large negative enthalpy change results from protein unfolding and changes in protein solvation.

1. Introduction

Urea is well known as a strong denaturant for unfolding the native conformation of globular proteins in aqueous solution [1]. The phenomenon is attributed to the perturbing effect of urea on the structure of water, which enhances the capacity of water to accommodate hydrophobic groups of the protein [2]. A theoretical analysis of the ability of urea to act as a water structure breaker has been reported [3]. It has also been suggested that urea interacts directly with the protein – specifically, that the amine groups in urea complex with the carbonyl groups of the polypeptide backbone by hydrogen bonding – and that this interaction is a necessary contribution to the denaturing process, even though it is not a predominant factor [4].

The protein phycocyanin, which is an accessory pigment in photosynthesis, exists in solution as several different aggregates. Hydrophobic interaction plays an essential role in its aggregation. We have recently employed microcalorimetry to study the thermodynamic aspects of the interaction of phycocyanin with tetraalkylammonium bromides [5].

The present investigation is an extension of our studies on protein aggregation and protein–salt interaction [5,6]. Microcalorimetry, UV and visible spectra, circular dichroism (CD) and sedimentation equilibrium were employed to investigate the denaturation of phycocyanin in urea solutions. The enthalpy (ΔH) of the protein denaturation was measured by microcalorimetry. The possible major contributions to the enthalpy change during the denaturation are discussed.

2. Materials and methods

Phycocyanin from the blue-green alga *Phormidium luridum* was extracted and purified as described previously [6]. Sodium phosphate buffer at pH 6.0 and an ionic strength of 0.1 was used in preparing the protein solutions. Phycocyanin solutions containing only trimer and hexamer were prepared by dialyzing phycocyanin solutions into pH 8.7 solution (prepared from pH 8.1 sodium phosphate-NaOH buffer adjusted with additional NaOH) overnight and then dialyzing back to pH 6.0. This method is effective in eliminating the presence of higher aggregates of phycocyanin.

Urea (ultra pure grade from Schwarz/Mann Co.) was purified by treatment with a mixed ion exchange resin (AG-501-X8 analytical grade, Bio-Rad Laboratories) [7]. The purity of the urea solution effluent from the ion exchange column was monitored using a type

CDM2e conductivity meter (Radiometer Laboratories). Aliquots of the pure urea solution were evaporated to dryness and stored in a vacuum desiccator.

Ultraviolet and visible absorption spectra and CD of phycocyanin in the presence and absence of urea were measured with a Cary model 14 spectrophotometer and a Cary model 61 CD spectropolarimeter, respectively.

A twin-reaction-cell batch microcalorimeter was used to measure the heats of mixing. The instrumentation, calibration and experimental procedures have been described previously [5]. The measurements were carried out at 25°C. The scheme for the protein–urea mixing experiments was as follows: The two compartments of the reaction cell contained 0.700 ml of an appropriate concentration of urea in phosphate buffer, pH 6.0, and 0.200 ml of phycocyanin solution (10 mg/ml in phosphate buffer). The compartments of the reference cell contained the same concentration and volume of urea solution and 0.200 ml of phosphate buffer.

Sedimentation equilibrium and velocity measurements were performed at 25°C on a Spinco model E ultracentrifuge equipped with an electronic speed control and photoelectric scanner. The speed was 40 000 rpm for the equilibrium runs and 60000 rpm for the sedimentation velocity runs. The partial specific volume ($\bar{v}$) of 0.75 ml/g of phycocyanin in buffer solution [8] was used to compute the molecular weight of the protein in 3–8 M urea solutions. In the presence of 8 M urea a 1% decrease in $\bar{v}$ has been reported for other proteins [9], and the presence of such a small change in $\bar{v}$ affects the calculated molecular weight to the magnitude of about 600 out of 14300. This uncertainty does not have any effect on the interpretation of the results.

The densities of urea in aqueous solution [10] are used as those in the buffer solution for the sedimentation equilibrium measurements. The difference between these two densities is negligible in the urea concentration ranges studied. For instance, the densities of 7 and 8 M urea in the buffer solutions were measured pycnometrically at 25 ± 0.01°C; it was found that the densities are 1.1193 ± 0.0001 and 1.1101 ± 0.004 g/ml, as compared to 1.1200 and 1.1045 g/ml in aqueous solutions, respectively.

3. Results

The UV and visible spectra of phycocyanin show a maximum absorption at 625 nm in phosphate buffer, pH 6.0, in the absence of urea. The maximum shifts to 620 nm in the presence of 1 M urea and to 615 nm in 2–6 M urea. The absorption at around 615 nm becomes broader in 7–10 M urea (fig. 1). The optical density (OD) at the maximum absorption decreases as the concentration of urea in the protein solution increases. A plot of molar extinction coefficient at the visible absorption maximum of phycocyanin as a function of the concentration of urea is presented in fig. 3A.

Circular dichroism measurements demonstrate that the strong positive dichroic band of phycocyanin at around 630 nm shifts to 600 nm at 2–10 M urea, while the strong negative band at 330 nm remains essentially unaltered (fig. 2). At 222 nm the molar ellipticity [θ] in the absence of urea is 21 deg cm^2/decimole. This value remains unchanged in the presence of 1–4 M urea, decreases in 5–7 M urea and remains essentially of the same magnitude in 8–10 M urea. The dependence of molar ellipticity of CD of phycocyanin at 222 nm on the concentration of urea is plotted in fig. 3B.

For a two-state mechanism in protein denaturation,

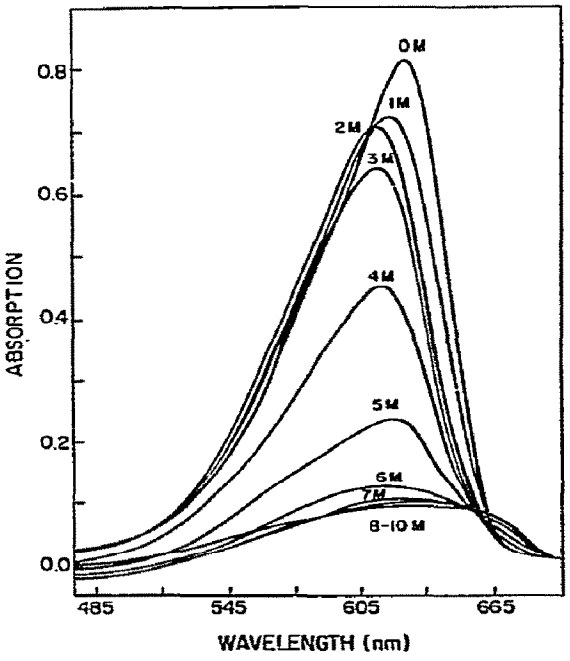

Fig. 1. Visible absorption spectrum of phycocyanin as a function of urea concentration (M). Phycocyanin concentration = 0.26 mg/ml. Path length = 0.5 cm.

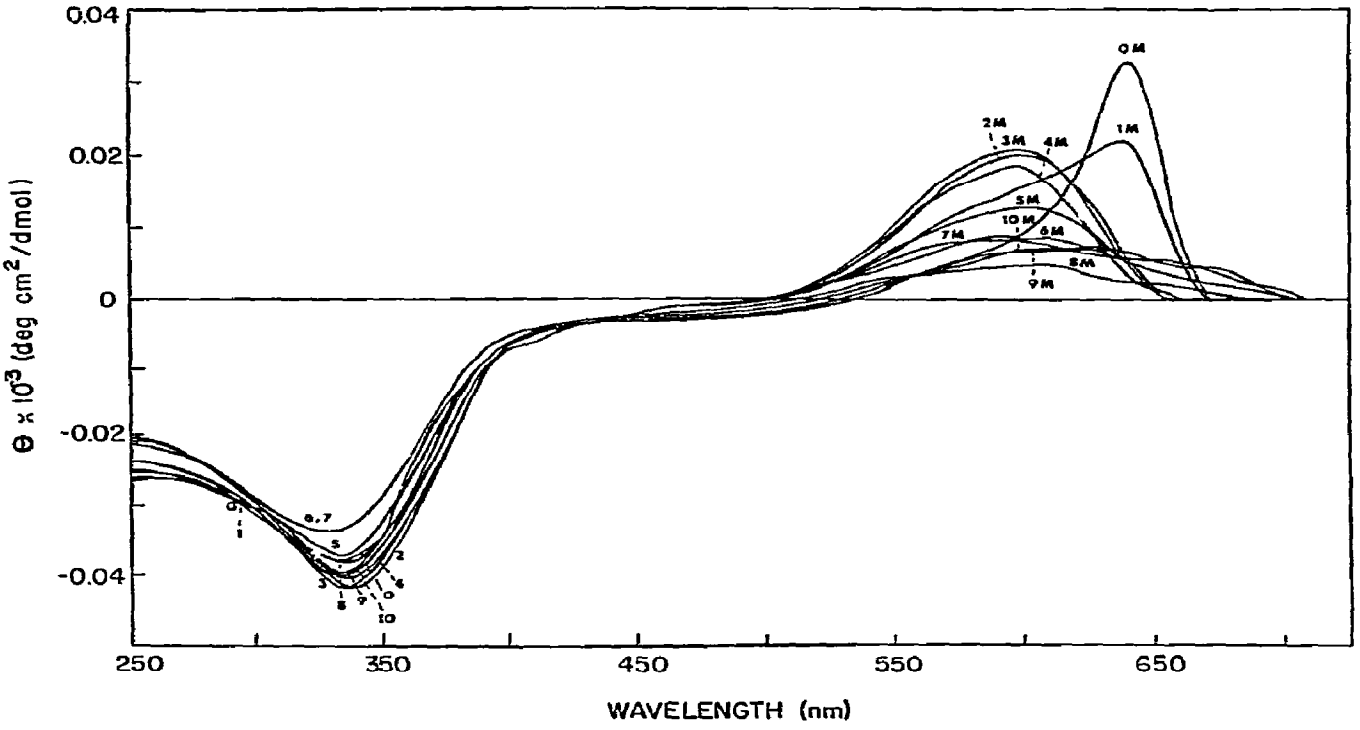

Fig. 2. CD spectrum of phycocyanin as a function of urea concentration (M). Phycocyanin concentration = 0.26 mg/ml.

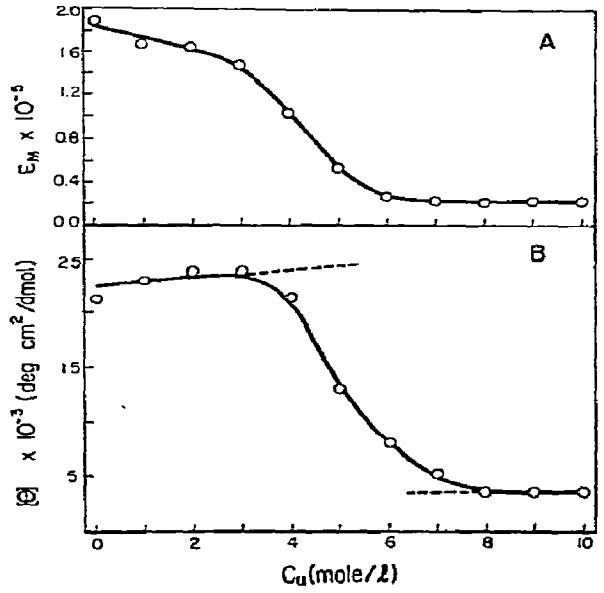

Fig. 3. (A) molar extinction coefficient (ϵ_M) at the visible absorption maximum and (B) molar ellipticity of CD at 222 nm ($[\theta]_{222}$) of phycocyanin as a function of the concentration of urea (C_u). Phycocyanin concentration = 0.26 mg/ml.

the equilibrium constant, K, and the free energy of denaturation, ΔG_{app}, can be determined from the experimental data by using

$$K = ([\theta]_N - [\theta])/([\theta] - [\theta]_D), \quad (1)$$

where $[\theta]$ is the observed molar ellipticity at 222 nm and $[\theta]_N$ and $[\theta]_D$ denote the molar ellipticities which the native and denatured states would have under the same conditions. A plot of the calculated ΔG_{app} for the unfolding of phycocyanin against C_u is shown in fig. 4. Fig. 4 demonstrates that ΔG_{app} varies linearly with denaturant concentration. A linear extrapolation of ΔG_{app} to zero urea concentration leads to a value of 4.4 kcal/mole for $\Delta G_{app}^{H_2O}$ at pH 6.0 and 25°C, as compared to 9.7 kcal/mole for ribonuclease at pH 6.6, 6.1 kcal/mole for lysozyme at pH 2.9, 8.3 kcal/mole for α-chymotrypsin at pH 4.3 and 11.7 kcal/mole for β-lactoglobulin at pH 3.2 [11].

Sedimentation equilibrium measurements employing the meniscus depletion method [12] show that the protein solution is apparently monodispersive in 4–8 M urea solutions. The straight-line plot of ln OD versus

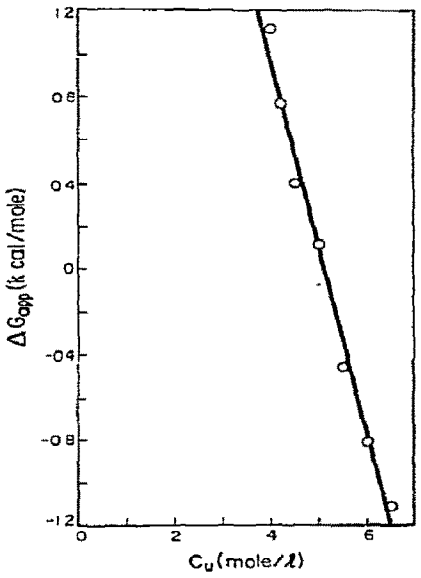

Fig. 4. Apparent free energy of unfolding, ΔG_{app}, as a function of urea concentration (C_u).

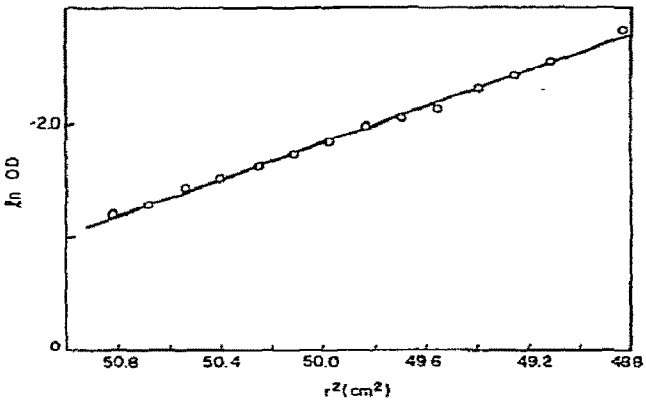

Fig. 5. Typical plot of ln OD versus r^2 by the meniscus depletion method of the sedimentation equilibrium measurement. Concentrations: phycocyanin = 0.8 mg/ml; urea = 8 M. λ = 280 nm; intercept = −42.07 ± 0.74; slope = 0.803 ± 0.015.

r^2 and the least-squares coefficients of the straight line are given in fig. 5. In these experiments OD is the optical density of the protein solution at 280 or 620 nm and the absorption is demonstrated to be a linear function of protein concentration (obeys Beer's law) and r is the distance to the center of rotation. The molecular weights of phycocyanin determined in 3–8 M urea solution are presented in table 1.

The phycocyanin–urea mixing reaction in the reaction cell of the microcalorimeter is

$$PC(M_{pc}, V_{pc}) + U(M_u, V_u) \rightarrow PC(C_{pc}, V_t), U(C_u, V_t). \quad (A1)$$

PC and U denote phycocyanin and urea, respectively. M_{pc} and M_u represent the concentrations of phycocya-

Table 1
Monomer and subunit molecular weight of phycocyanin from *Phormidium luridum*

Solution condition	Monomer mol. wt.	Subunit mol. wt. α	β	mean
no denaturation agent [a]	30000			
SDS + β-mercaptoethanol [b]		11900	18500	
urea solutions [c]				
8 M urea				13960 ± 230
7 M urea				14310 ± 270
6 M urea				13100 ± 130
5 M urea				13510 ± 280
4 M urea				13470 ± 210
3 M urea [d]	27100 ± 864			13130 ± 720

[a] From ref. [18]. [b] From ref. [20].
[c] Determined by sedimentation equilibrium measurements.
[d] A mixture of monomer and subunits.

nin (mg/ml) and urea (mole/l) before mixing. V_{pc} and V_u represent the volumes of phycocyanin (0.200 ml) and urea (0.700 ml), respectively. C_{pc} and C_u denote the concentrations of phycocyanin and urea after mixing. Thus $V_t = V_{pc} + V_u$; $C_{pc} = M_{pc}V_{pc}/V_t$ and $C_u = M_uV_u/V_t$.

The mixing reaction in the reference cell is

$$U(M_u, V_u) + \text{buffer}\ (V_b) \rightarrow U(C_u, V_t), \qquad (A2)$$

where $V_b = V_{pc}$.

Since the heats of dilution of urea in the reaction and the reference cells are effectively balanced out, the heat change (ΔQ_{mix}) observed in reaction (A1) is

$$\Delta Q_{mix} = \Delta Q_m + \Delta Q_{dil(pc)}, \qquad (2)$$

where ΔQ_m is the heat of reaction and $\Delta Q_{dil(pc)}$ is the heat of dilution of 0.200 ml of 10 mg/ml protein solution diluted with buffer solution to a final volume of 0.900 ml. Since the value of $\Delta Q_{dil(pc)}$ is negligible (less than 1 mJ) [5] in comparison with that of ΔQ_{mix}, ΔQ_{mix} can be presumed to be ΔQ_m, and the enthalpy of reaction (ΔH_{rn}) can be easily calculated from ΔQ_{mix}.

Values of M_u, C_u, M_{pc}, C_{pc}, ΔQ_{mix} and ΔH_{rn} are presented in table 2. Each value of ΔQ_{mix} is an average of two to four measurements. The plot of ΔH_{rn} versus C_u in fig. 6 demonstrates that in the denaturation range there is a sharp increase in the negative enthalpy. The same behavior has been observed in the denaturations of lysozyme in guanidine hydrochloride solution [13] and of chymotrypsin A by urea [14]. By extrapolating the linear part of the curve before (region I) and after (region II) the denaturation and drawing a vertical line at the midpoint of denaturation [13], the overall enthalpy of denaturation (ΔH_{dn}) can be estimated from the length of the vertical line to be −230 kcal/mole.

Table 2
Calorimetric results from mixing phycocyanin with urea in phosphate buffer, pH 6.0, I 0.1. Phycocyanin concentration: M_{pc} = 10 mg/ml; C_{pc} = 2.2 mg/ml [a]

Urea concentration		$-\Delta Q_{mix}$	$-\Delta H_{rn}$
M_u (mole/ℓ)	C_u (mole/ℓ)	(mJ)	(kcal/mole of monomer pc [b])
10.30	8.0	132.8 ± 4.5	476
9.64	7.5	125.0 ± 5.0	448
9.00	7.0	127.4 ± 4.0	457
7.71	6.0	81.1 ± 0.3	290
6.43	5.0	42.0 ± 3.8	151
5.14	4.0	35.0 ± 0.3	125
3.86	3.0	28.5 ± 0.4	102
2.57	2.0	14.5 ± 0.1	52
1.285	1.0	7.6 ± 3.8	27

a) For definitions of terms, see reaction equation (A1).
b) ΔH_{rn} = [ΔQ_{mix}(mJ)/4.185 (J/cal)]/[M_{pc}(mg/ml) × 0.200 ml]/30000 (mol. wt. of monomer pc)].

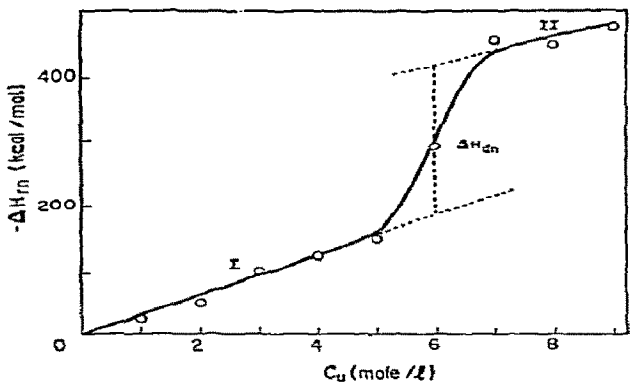

Fig. 6. ΔH_{rn} as a function of urea concentration (C_u).

4. Discussion

The OD or molar extinction coefficient of the visible absorption spectrum decreases as the concentration of urea increases to 7 M and then levels off (fig. 1, fig. 3A). The essentially constant visible absorption spectrum at $C_u \geq 7$ M can be interpreted as evidence that the protein is completely denatured. This finding is consistent with the minimum changes in $[\theta]$ in the 500–650 nm region at $C_u > 7$ M (fig. 2, fig. 3B). The CD results also suggest that the protein conformation change starts at $C_u > 4$ M and is completed at C_u = 7–8 M.

The complex interaction of the chromophore with the protein and solvent is primarily responsible for both the position and the relative intensity of phycocyanin's maximum absorption at 620–630 nm. The shift of this maximum to a lower wavelength and the decrease in its relative intensity in visible and CD spectra in the presence of 2–10 M urea indicate that the

chromophore–protein solvent interaction is modified as a result of the new environment containing denaturant.

The values of $[\theta]$ obtained by CD at 190–250 nm have been used to compute the relative contents of the helix (α), the β form and the unordered form of the protein [15]. The $[\theta]$ of a protein at a specific wavelength can be represented as

$$\theta = f_\alpha \theta_\alpha + f_\beta \theta_\beta + f_R \theta_R, \quad (3)$$

where θ_α, θ_β and θ_R are the reference molar ellipticities for the α, β and unordered forms of the protein. f_α, f_β and f_R are the fractions of the α, β and the unordered forms. The values of θ_α, θ_β and θ_R at 222 nm are –31500, –4000 and –2000 deg cm^2/decimole, respectively [14]. By employing these data, eq. (3) and the values of θ appearing in fig. 3B, it can be roughly estimated that the protein contains about 65% of α helix at $C_u = 0$ and $< 10\%$ at $C_u > 7$ M. This indicates that the protein undergoes very significant conformation changes (unfolding) during urea treatment.

The CD spectra of the separated α and β subunits of phycocyanin from different algal sources, *Synechococcus* sp. and *Cyanidium caldarium*, have been examined [16,17]. At pH 7.0 the CD spectra display an absorption maximum at 620 nm for the α subunit and 608 nm for the β subunit in *Synechococcus* sp. and 620 nm for α subunit and 585 nm for the β subunit in *Cyanidium caldarium*. Our data show a broader absorption at 600 nm for the mixture of α and β subunits in 3–10 M urea.

In 4–8 M urea, the determined mean molecular weight of the subunits is about 14000 daltons (table 1) which is not far from one-half of the monomer molecular weight of phycocyanin (30000) [18]. This result, in view of the monodispersion shown by sedimentation equilibrium measurements, suggests that the protein is completely denatured into its subunits at 4–8 M urea concentrations and that the two subunits have relatively close molecular weights. A mixed molecular weight, 13130 close to the meniscus and 27100 close to the cell bottom, is found in 3 M urea solution. This indicates that in 3 M urea the protein contains a mixture of monomer and subunits.

The value of –230 kcal/mole (ΔH_{dn}) at $C_u = 6$ M assigned for the enthalpy of denaturation (or the overall enthalpy of unfolding) is comparable with –300 kcal/mole for the denaturation of lysozyme in guanidine hydrochloride solution [13]. This relatively large negative enthalpy of denaturation contains contributions from protein unfolding and changes of protein solvation. A method has been presented for calculating the number of guanidine hydrochloride and water molecules bound to protein in 6 M guanidine hydrochloride solution [19]. However, in the present case, without knowing the state of protein solvation as a function of urea concentration and the hydrophobic interactions of protein, it is not possible to make any relevant estimation of the individual contributions.

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