Biophysical Chemistry, 34 (1989) 155–162
Elsevier

BIOCHE 01388

Calorimetric and circular dichroic studies of the thermal denaturation of β-lactoglobulin

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Received 1 May 1989
Revised manuscript received 3 July 1989
Accepted 3 July 1989

β-Lactoglobulin; Protein conformation; Thermal denaturation; Calorimetry; Circular dichroism

The thermal denaturation of β-lactoglobulin in aqueous solutions at pH 5.5 and 2.0 was investigated by differential scanning calorimetry (DSC) and circular dichroic (CD) measurements. By calorimetry, the denaturation temperatures (T_d), denaturation enthalpies, and specific heat capacity changes accompanying denaturation have been obtained. This allowed calculation of the enthalpy, entropy, and Gibbs free energy changes for thermal denaturation in the temperature range scanned, i.e., 20–100 °C. The unfolding process was found to be only partially reversible. Analysis of the far-ultraviolet CD spectra reveals that with increasing temperature the mean residue ellipticity ($[\theta]$) becomes less negative, which reflects unfolding of the native protein. At the highest temperature of CD measurements, i.e., 80 °C, conformational changes are to a large extent reversible.

1. Introduction

In a previous paper [1], the denaturation of β-lactoglobulin by urea and alkylureas was reported. The methods used were batch calorimetry and circular dichroism (CD). The former gives rise to heat effects accompanying denaturation, while the latter reflects conformational changes. The main conclusion based on a comparison of the denaturing action of urea and alkylureas, respectively, was that the differences ascertained reflected the presence of the hydrophobic moiety in the urea molecule. The subject of this investigation was to examine by differential scanning calorimetry (DSC) and CD the thermal denaturation, i.e., the denaturing action of heat, of β-lactoglobulin in aqueous solution. The phenomenon has been extensively studied by using various methods, e.g., optical rotatory dispersion [2,3], light scattering [4,5], electrophoresis [6], chromatography [7], and DSC [8–10]. The results obtained with these methods reflected changes in various physicochemical properties brought about by heating. Thus, by DSC, the denaturation enthalpy ΔH_d and denaturation temperature T_d have been ascertained. The latter is the temperature of the maximum heat absorption.

However, for a complete thermodynamic description of thermal denaturation not only ΔH_d and T_d but also the denaturation Gibbs free energy ΔG_d and entropy ΔS_d as well as their temperature dependence are needed. The thermodynamic data can be calculated from the difference in heat capacity of the denatured and native protein that can be obtained from DSC curves by a graphic analysis [11–13]. Since for β-lactoglobulin these data were unavailable, DSC was applied in order to obtain them.

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Bovine β-lactoglobulin is usually a mixture of variants A and B. The two variants differ at positions 64 and 118, where an aspartic acid and a valine in variant A are substituted by a glycine and an alanine in variant B. Both forms have two disulfide bridges and a free sulfhydryl group which explains their tendency to undergo association (reversible) and aggregation (irreversible) [14] (see section 2). The monomer has a molecular weight of 18400 and a single polypeptide chain of 162 amino acid residues. X-ray diffraction studies reveal that there are several crystal forms of β-lactoglobulin, e.g., trigonal and orthorhombic, due to differences in packing [15–17]. Variants A and B, separate or mixed, crystallize isomorphously in the trigonal as well as the orthorhombic forms.

A few words about the association and aggregation of β-lactoglobulin in aqueous solution are in place. At pH 2 and room temperature, it is predominantly in the monomeric form, whereas at pH approx. 7 it exists as the dimeric species [18–21]. By increasing the temperature the equilibrium is shifted towards the monomeric form. At 55 °C, even at neutral pH, the dimers (or rather oligomers) are almost completely dissociated [2]. At higher temperatures, i.e., above 80 °C, unfolded species that associate appear. At still higher temperatures, aggregation that involves the sulfhydryl and disulfide groups sets in [6].

Since an important aspect of any denaturation is its reversibility, separate experiments have been performed with cooled, previously denatured samples. Both methods applied in this study, DSC and CD, were used for this test.

2. Experimental

Bovine milk β-lactoglobulin B was kindly supplied by Dr. C.N. Pace (Texas A&M University). The mixture of A and B variants was obtained from Sigma (St. Louis, MO) as a three-times crystallized and lyophilized powder that was used without further purification.

Protein was dissolved either in water (pH 5.5) or in 0.1 M glycine/0.1 M HCl (pH 2.0). Its concentrations were determined by using $E_{1\,cm}^{1\%\,w} = 9.6$ at 278 nm.

Calorimetric experiments were carried out with a Bio-DSC batch microcalorimeter from Setaram (Caluire, France). The calorimetric block has the temperature controlled with a very high accuracy. The sample and reference vessels are located within two bores inside the block. Two heat-flux transducers surround the vessels, linking them to the block. By means of a feedback system, the vessels are maintained at identical temperatures throughout a temperature-scanning experiment. The heat supplied to the reference or sample vessel in order to keep them balanced is measured. The heat capacity of the sample solution relative to that of the reference solution is directly recorded vs. the temperature. The vessel volume is 1 ml. Calibration of the calorimeter is performed with special vessels by using the Joule effect. The duration and cycle of each pulse can be preset. A constant current supply is generally used so that it is only necessary to measure the resulting voltage to determine the power applied to the sensor.

In calorimetric experiments the mixture of variants A and B was used. Owing to the small difference in composition mentioned above, the specific heat capacities of the two variants are practically equal. The protein concentration was between 1 and 2%. The solution volume in experiments was 0.7 ml. DSC curves were recorded over the temperature range 20–100 °C. The heating rates in DSC experiments were 0.5 and 1.0 °C/min. A few experiments were performed at 1.5 °C/min which is the highest rate attainable with this microcalorimeter.

For each protein concentration and heating rate, at least three experiments were performed. Amplification applied in the experiments was 50 μV (500 μW) and 100 μV (1 mW) full-scale deflection (f.s.d). The maximum amplification of the instrument is 1 μV (10 μW). However, owing to imperfect matching of the vessels, operation at maximum sensitivity is not feasible. Namely, blank experiments with equal weights of solvent in both vessels revealed that they are not thermally balanced. The baselines that were obtained were asymmetric curves with clearly expressed minima. This signifies that there arise disturbances from the surroundings which are not common to both vessels. Moreover, the higher the amplification,

the larger is the deviation from a straight line. Thus, as mentioned above, the maximum amplification applied was 50 μV. Proof that the difference signal in blank experiments is due to perturbations stemming from the surroundings was provided by experiments with stainless-steel, dummy vessels. The recorded curves resemble those obtained in blank experiments, i.e., with equal weights of solvent in both vessels.

The thermograms registered in experiments with protein solutions were corrected for 'nonideality' in the following way. From each thermogram, the curve of the blank experiment was subtracted. The problem that arose was the baseline of the blank experiment. Fortunately, at low temperatures (5–15 °C), blank thermograms are linear. Assuming now, as a first approximation, that the baselines are straight lines throughout the entire temperature range examined, the corrections for nonideality became feasible. The corrected thermograms do not differ from those reported by others in similar experiments [11,13] (cf. fig. 1). Moreover, the procedure was checked by repeating the experiments of Jackson and Brandts [11] who studied the thermal denaturation of chymotrypsinogen. The values of ΔH_d obtained differed from those reported by the aforementioned authors by less than 10%.

CD spectra were recorded with a mark III Roussel-Jouan dichrograph as described previously [1]. The mean residue ellipticity $[\theta]$ was calculated by using the following equation

$$[\theta]_{mrw} = \frac{M_0 \theta}{100 cl} \quad (1)$$

where M_0 denotes the mean residue molecular weight (114), based on a molecular weight of 18 400, θ the ellipticity, c concentration (in g/cm^3) and l path length (in dm). For CD measurements the variant B was used. The solvents were the same as in calorimetric experiments.

The highest temperature at which CD spectra were recorded was slightly above 80 °C. The instrumental setup used did not allow measurements at higher temperatures. In the experiments silica cells of 0.05 cm path length were used.

3. Results and discussion

3.1. Differential scanning calorimetry

In fig. 1 a typical corrected thermogram for determining the enthalpy of denaturation at the temperature of the thermogram maximum, ΔH_d^m, and the difference in specific heats of the denatured and native protein, ΔC_p^d, is presented.

It is a relatively symmetrical curve with a well-expressed peak, i.e., the maximum of heat absorption. The area of the peak is taken as the area limited from above by the thermogram and from below by the heat capacity lines of the native C_p^n and denatured protein C_p^d obtained by linear extrapolation to the midpoint of the transition. Thus, thermal denaturation is assumed to be a two-state transition involving only the native and denatured states [11–13]. The areas obtained in this way were cut off and weighed. Comparison with areas (or rather weights) obtained in calibration experiments under identical experimental conditions yielded values of ΔH_d^m.

Further examination of fig. 1 reveals that the values of C_p^n and C_p^d are independent of temperature which does not hold true [11–13]. The heat

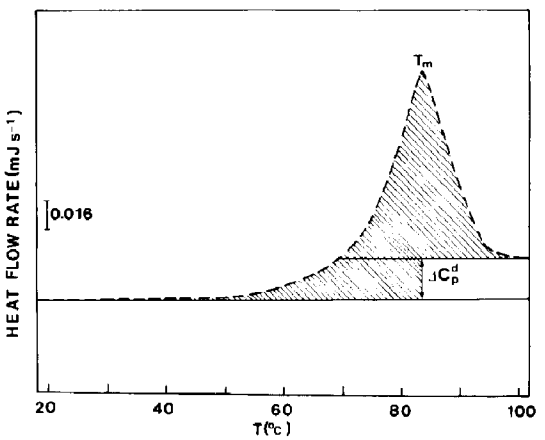

Fig. 1. Heat absorption curve of an aqueous solution of β-lactoglobulin after correction for the baseline. ΔC_p^d designates the difference in specific heat capacity between the denatured (C_p^d) and native (C_p^n) protein. The hatched area represents the denaturation enthalpy ΔH_d^m. For details, see text.

capacities are temperature-dependent but the change with temperature is small and is undetectable under the existing experimental conditions. The difference though, ΔC_p^d, is independent of temperature. Its value obtained from corrected thermograms is 0.288 ± 0.03 J/K per g and does not depend on pH [13]. Most calorimetric experiments were performed at pH 2.0, only a few being carried out at pH 5.5.

From known values of ΔH_d^m, T_d^m and ΔC_p^d, the values of ΔH_d, ΔS_d and ΔG_d within the temperature range 20–95 °C have been calculated. The following equations were used [13,22]

$$\Delta H_d^T = \Delta H_d^m - \int_T^{T_m} \Delta C_p^d \, dT$$

$$= \Delta H_d^m - \Delta C_p^d (T_m - T)$$

$$\Delta S_d^T = \frac{\Delta H_d}{T_m} - \int_T^{T_m} \frac{\Delta C_p^d}{T} \, dT = \frac{\Delta H_d^m}{T_m} - \Delta C_p^d \ln \frac{T_m}{T} \quad (2)$$

$$\Delta G_d^T = \Delta H_d^T - T\Delta S_d^T$$

In fig. 2 the three quantities, ΔH_d, $T\Delta S_d$ and ΔG_d, are presented as functions of temperature. In table 1 the values of the same quantities in the temperature range 20–95 °C are listed. These are the so-called stabilization parameters referring to the transition of a native protein to a randomly coiled form [22]. Let us now examine the results in table 1 more closely. Especially interesting are the values of individual quantities at T_d^m and at 'standard' temperature, 25 °C. At T_d^m, ΔG_d is naturally zero, since this is the temperature of the midpoint of the transition. At pH 5.5, T_d^m is 80.0 °C and ΔH_d^m amounts to 412 kJ/mol which is also the value of $T\Delta S_d$. At 25 °C, the value of ΔH_d is 121 kJ/mol, $T\Delta S_d$ is 81 kJ/mol and ΔG_d amounts to 40 kJ/mol.

At pH 2.0, T_d^m is 83.2 °C, whereas ΔH_d and $T\Delta S_d$ have the same value, 414 kJ/mol. At 25 °C, ΔH_d is 106 kJ/mol, $T\Delta S_d$ 65 kJ/mol, and ΔG_d 41 kJ/mol. The values of the denaturation quantities in table 1 are generally comparable to those of other globular proteins [13]. The fact that down to 20 °C the values of ΔG_d increase and those of $T\Delta S$ decrease indicates that the temperature of

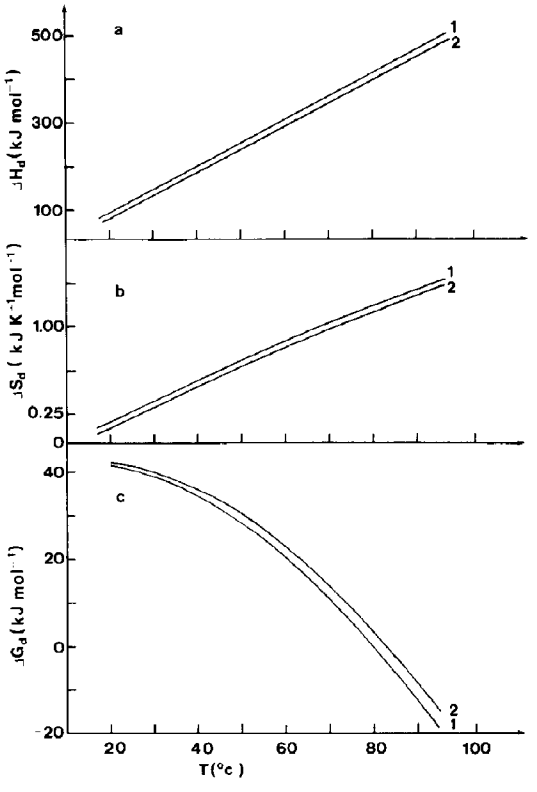

Fig. 2. Temperature dependence of denaturation enthalpy, ΔH_d, entropy, ΔS_d, and Gibbs free energy, ΔG_d. (1) pH 5.5, (2) pH 2.0.

maximum stability is below 20 °C which has also been found, for example, with α-chymotrypsin, which has a relatively similar secondary structure to that of β-lactoglobulin [13]. The positive differences between the values of ΔG_d at pH 2.0 and 5.5, respectively, as well as the higher T_d^m at the lower pH could reflect the increased stability of β-lactoglobulin at pH 2.0.

As mentioned in section 2, the heating rates in the calorimetric experiments were 0.5, 1.0 and 1.5 °C/min. The values of T_d^m and ΔH_d^m ascertained differed within the limits of experimental error (15%). It is well known that a strong dependence of T_d^m on the heating rate very likely indicates an irreversible process or a rate of reaction comparable to that of scanning [23]. Therefore, this finding suggests that there is at least partial reversibility. We shall return to this problem after

Table 1

Thermodynamic quantities (in kJ/mol) of thermal denaturation of β-lactoglobulin (enthalpy, ΔH_d; entropy × T, $T\Delta S_d$; Gibbs free energy, ΔG_d) at pH 5.5 and 2.0

In all calculations, the molecular weight of β-lactoglobulin was taken to be 18400.

T (°C)	pH 5.5			pH 2.0		
	ΔH_d [a] (kJ/mol)	$T\Delta S_d$ [a] (kJ/mol)	ΔG_d [a] (kJ/mol)	ΔH_d [a] (kJ/mol)	$T\Delta S_d$ [a] (kJ/mol)	ΔG_d [a] (kJ/mol)
20	94.5	53	41.5	80	38	42
25	121	81	40	106	65	41
30	148	109	39	133	93	40
35	174	137	37	159	121	38
40	201	166	35	186	150	36
45	227	196	31	212	179	33
50	253	225	28	239	209	30
55	280	256	24	265	238	27
60	306	286	20	292	269	23
65	333	317	16	318	299	19
70	359	348	11	345	331	14
75	386	380	6	371	362	9
80	412	412	0	398	394	4
85	439	445	−6	424	426	−2
90	565	478	−13	450	459	−9
95	492	511	−19	477	492	−15

[a] Relative error is estimated to be about 10% at T_d increasing to about 15% at room temperature.

discussing the calorimetric results obtained by other authors and the CD data.

Rüegg et al. [9] found T_d^m to be 72.8°C and ΔH_d (227 ± 21) kJ/mol; the values for the pH were 6.66 (20°C) and 6.42 (80°C). De Wit and Swinkels [10] determined ΔH_d^m at T_d^m 71°C and pH 6.7 to be (230 ± 15)kJ/mol. Thus, in both cases the values of T_d^m and ΔH_d^m are much lower than those obtained in this study. The difference could be due to the fact, firstly, that the above-mentioned authors used small amounts of rather concentrated solutions so that, owing to aggregation, unfolding was incomplete. Secondly, their procedure for the evaluation of thermograms may involve a larger error than assumed owing to incomplete baseline correction. Very recently, values of T_d^m and ΔH_d^m at pH 2.0 have been obtained by DSC that are very close to those found in this investigation (P.L. Mateo, personal communication).

Let us now comment on the values of ΔH_d at 25°C. At pH 5.5, the value is 121 kJ/mol and at pH 2.0 it amounts to 106 kJ/mol. In a previous study [24], the stabilization enthalpy of β-lactoglobulin at 25°C and neutral pH has been estimated to be 188 kJ/mol. The value of 121 kJ/mol determined in this study, although much smaller, may be considered more reliable. For ΔG_d no other calorimetric values appear to be available. However, values of ΔG_d exist based on optical rotation [25] and ultraviolet difference spectroscopy [26]. The value obtained by optical rotation at 25°C and pH 3.2 is 49 kJ/mol which agrees satisfactorily with those values obtained in the present study, viz., 40 and 41 kJ/mol at pH 5.5 and 2.0, respectively. The determination is based on the two-state mechanism for denaturation in urea and guanidine hydrochloride. ΔG_d has been obtained as a function of denaturant concentration. The ultraviolet spectroscopy data were also analyzed on the basis of a two-state approximation to obtain the apparent equilibrium constant, K_{app}, for protein unfolding as a function of temperature [26]. From K_{app}, calculations of ΔG_d, ΔH_d and ΔS_d as functions of temperature and pH have been carried out. The values found are comparable to those determined in this study. However, the results indicate a strong dependence

of the thermodynamic quantities on pH which has not been observed in DSC studies. The ultraviolet absorbance data are based by and large on the behavior of two tryptophan residues out of 162 in the whole molecule. The question now arises as to whether the data based on tryptophan reflect the overall conformational change. The calorimetric results show that this is not the case. The problem has been discussed in detail elsewhere [27]. A recent warning in the same connection is based on experiments with α-lactalbumin for which the degree of unfolding as determined by nonthermodynamic methods (CD) does not seem to be relevant to calorimetrically determined thermodynamic quantities [28].

3.2. Circular dichroism

The CD spectra of β-lactoglobulin B were recorded for solutions at pH 5.5 and 2.0. They are presented in figs. 3 and 4. The main feature of the spectra is the decreasing values of $[\theta]$ (less negative) with increasing temperature. Moreover, the values of $[\theta]$ at pH 2.0 are only slightly more negative than those at pH 5.5. This is in accord with the fact that β-lactoglobulin shows a remarkable stability to low pH even at pH 2.0 [29]. The value of $[\theta]$ as well as its change at high temperature depends on the protein structure. X-ray diffraction studies [15–17] show that β-lactoglobulin consists of antiparallel β-sheets formed by nine β-strands accounting for approx. 50% of the peptide residues, about 10% of α-helix, 15% of reverse turns, and the rest unordered. Considering the contributions of individual forms, the overall value of $[\theta]$, being the sum of contributions, is relatively small which also applies to its change on (thermal) denaturation [30]. The conclusions are confirmed by a comparison of thermal denaturation with that induced by urea (fig. 3) (S. Lapanje and N. Poklar, unpublished data). The values of $[\theta]$ also decrease with increasing urea concentration and in 8 and 9 M urea are considerably less negative than at 80 °C, the highest temperature at which CD spectra were recorded. With separate studies of CD at higher temperature it should be possible to ascertain whether the two denaturation processes give identical conformational states.

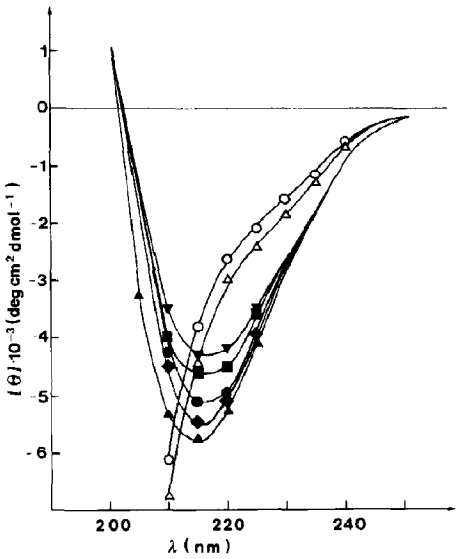

Fig. 3. Temperature dependence of far-ultraviolet CD spectra of β-lactoglobulin B in aqueous solution of pH 5.5: (▲) 28, (◆) 39, (●) 48, (■) 58, (▼) 81 °C. For comparison, the CD spectra of β-lactoglobulin B in 8 M (△) and 9 M (○) urea at 25 °C are included.

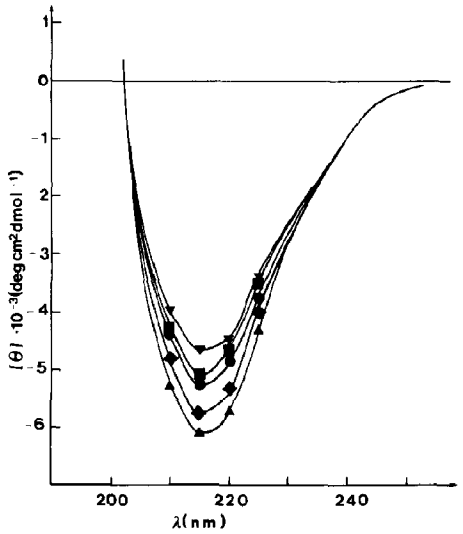

Fig. 4. Temperature dependence of far-ultraviolet CD spectra of β-lactoglobulin B in aqueous solution of pH 2.0: (▲) 26, (◆) 37, (●) 46, (■) 57, (▼) 73 °C.

Unfortunately, the problem is not that simple. As already mentioned, at temperatures above 80 °C one has to reckon with various types of aggregation [6].

This leaves us with the problem of the reversibility of the thermal denaturation of β-lactoglobulin. To estimate renaturation of the protein after thermal denaturation, previously denatured samples were cooled in the calorimeter vessel at a rate of 5 °C/min and rescanned. Protein solutions of pH 5.5 showed little renaturation. The thermogram was extended and had a poorly expressed peak after correction for the baseline. On the other hand, protein solutions of pH 2.0 showed partial renaturation, the peak was still present and the usual process of evaluation was feasible. If the ratio of the peak areas is taken as a measure of the extent of renaturation, one obtains values of the ratio between 0.5 and 0.6. CD spectra of the renatured protein up to 80 °C are identical or very similar to those of the native protein. This shows that the conformation, even after heating to 80 °C and subsequent cooling, is to a large extent recovered.

These findings are not surprising on considering the complex processes occurring with increasing temperature and involving aggregation. The latter is clearly an irreversible process. Recent infrared studies up to 90 °C at pH 7 [31] indicate that β-lactoglobulin during thermal denaturation does not unfold to a random coil before aggregating and polymerizing towards the end of the process. The studies also show that after completion of the thermal denaturation at 90 °C, the infrared spectrum demonstrates the presence of bands due to β-sheets and α-helices and a very weak band due to random coils. The rigid globular structure is not completely unfolded by heat. This naturally shows how ambiguous terms like complete denaturation (unfolding) or random coils, for example, can be when referring to the unfolding process in thermal denaturation. At least some information regarding the structure that can be obtained by, e.g. infrared NMR or CD spectroscopy, is needed.

Summing up, it is possible to state that the thermal denaturation of β-lactoglobulin is a complex process. The values of the thermodynamic quantities determined in this study referring to the process are, due to its irreversibility, of limited value only. However, comparison of the values with those of other globular proteins for which reversibility exists shows that they are comparable. Therefore, the irreversibility from a thermodynamic point of view does not represent a major disturbance. For a detailed assessment of the species existing at individual temperatures at pH 2.0 and 5.5, the application of nonthermodynamic methods is indicated. The species could be produced by association or aggregation as well as by (partial) unfolding yielding structure-specific intermediates that can be detected and identified. Based on such knowledge, the mechanism of irreversibility could be illuminated.

Acknowledgement

This investigation was supported by a grant from the Slovene Research Community.

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