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Evaluation of carboxylic acid-induced conformational transitions of β-lactoglobulin: Comparison of the alcohol effects on β-lactoglobulin

Bong-Kuk Lee, Toshinori Shimanouchi, Hiroshi Umakoshi, Ryoichi Kuboi *

Department of Chemical Science and Engineering, Graduate of School of Engineering Science, Osaka University, 1-3 Machikaneyama Toyonaka, Osaka 560-8531, Japan

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Abstract

Conformational transitions of bovine β-lactoglobulin A (β-LG) induced by carboxylic acid were systematically studied by steady-state tryptophan (Trp) fluorescence. The behavior of β-LG denaturation depends upon the species and concentration of carboxylic acid, as well as on the pH of solutions. The order of the effectiveness of the respective carboxylic acids was described as follows: MeCOOH < EtCOOH < PrCOOH < ClEtCOOH < TFA < *i*BuCOOH < *n*BuCOOH < PFPA. The conformational change of β-LG through the carboxylic acid-induced transitions of the β-LG conformation were analyzed assuming a two-state mechanism between unfold and native states in order to obtain the *m* value, a measure of the dependence of the free energy change on the concentration of carboxylic acid. The *m* values of various carboxylic acids were compared with those of various alcohols based on the role of each group constituting the carboxylic acid and alcohol molecules, namely, the hydrocarbon group, hydroxyl group, halogen substituents, and the carboxyl group. Among these groups, the hydrophobic hydrocarbon groups and halogen substituents contributed positively to the *m* value, whereas the hydrophilic carboxyl and hydroxyl group contributed negatively. The present results can therefore be interpreted as a simple correlation based on the accessible surface area (ASA) of each groups of carboxylic acids and alcohols. These results suggest that the conformational transition of the protein due to the addition of carboxylic acids and alcohols can be explained both by hydrophobicity as well as clustering effects of each carboxylic acid and alcohol molecule.

Keywords: β-Lactoglobulin; Unfolding; Carboxylic acid; Alcohol; Accessible surface area; Cluster formation

1. Introduction

The functions of proteins are known to depend on their conformation under the given environmental conditions. The thermodynamic analysis of the protein conformation in the presence of particular solvents is an effective way to determine the stability, folding pathway, and intermolecular aggregation behavior of the proteins. In order to understand these mechanisms among various proteins, the conformational stability of proteins has previously been investigated using various methods, including the addition of acids [1,2], denaturants [3], solvents [4], or heat [5]. A number of studies have investigated the effects of organic solvents, especially alcohols, on the protein conformation in order to clarify the following phenomena: protein folding kinetics [6–9], the tuning of solvent conditions for the studies of the amyloid formation of peptides and proteins [10,11], the dissection and reassembly of amyloid fibrils [10,12], and the $\alpha \rightarrow \beta$ transition of proteins [13,14]. These effects of alcohols on the conformation of proteins can be explained by various properties of alcohols, namely, solvent polarity [15,16], dielectric constant [4], length of the carbohydrate chain, number of OH groups, degree of halogenation and cluster formation [17–19], and direct or indirect binding [20]. A novel approach to quantify the contribution of different alcohols on the conformational change

Abbreviations: ASA, accessible surface area; PFPA, pentafluoropropionic acid; Trp, tryptophan; C_m, midpoint concentration; β-LG, β-lactoglobulin; ΔG, Gibbs free energy

* Corresponding author. Tel.: +81 6 6850 6285; fax: +81 6 6850 6285.
E-mail address: kuboi@cheng.es.osaka-u.ac.jp (R. Kuboi).

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of polypeptides and proteins has recently been reported, where the additive contributions of particular groups of an alcohol molecule was calculated from the accessible surface area (ASA) [18,19,21].

Carboxylic acid molecules with a hydrophilic carboxyl (COOH) group have a similar structure to alcohol molecules possessing a hydrophilic hydroxyl (OH) group and have previously been utilized as a reagent to induce the conformational change of protein. In general, non-ionized carboxylic acid is known to function as an acidic organic solvent. Some acids, such as trichloroacetic acid, are routinely used as a protein-precipitating agent [22]. On the other hand, Yang and his coworkers have shown that the carboxylic acids suppressed the aggregation of proteins during their rapid refolding process [23]. Furthermore, the perfluorinated carboxylic acids such as trifluoroacetic acid (TFA) and pentafluoropropionic acid (PFPA) have been used to dissolve peptides and proteins [24,25]. Various carboxylic acids are known to denature proteins in the native state [26–28]. The acid treatment of proteins has reported to alter the activity of proteins [27], as well as their secondary and tertiary structures [28–30]. The effects of acid on the protein conformation have previously been explained by the balance between hydrophobic interactions and the affinity of anions for positively charged ammonium groups; such interactions and affinities could determine the conformation of acid-denatured proteins [31]. However, the exact mechanisms of the effects of carboxylic acid on proteins remain unclear because carboxylic acids also function as organic solvents. In our previous studies [32,33], it has been shown that the alcohol and carboxylic acid molecules could be the effective modifiers to control the property of reverse micellar interface. The addition of the alcohols and fatty acids also suppresses the formation of reverse micellar clusters remarkably and to enhance the back-extraction of proteins in the reverse micellar systems [34,35]. The quantitative analysis of the effects of alcohols and carboxylic acids on proteins is important because of its variety of potential applications.

The purpose of this study is to investigate the possible roles of various carboxylic acids on the conformational change of protein. Bovine β-lactoglobulin (β-LG) was used as a model protein. Although β-LG is known to exist as a dimer at a neutral pH, the native structure of β-LG has been reported to be stable in acidic environments as low as pH 2 [31,36–38]. In order to generalize the effects of carboxylic acids on this protein, the carboxylic acid-induced unfolding of β-LG was systematically investigated in terms of the species and concentrations of various carboxylic acids. In order to compare the effects of these carboxylic acids quantitatively, it was assumed that a two-state transition between the native and unfolded state could be applied during the carboxylic acid-induced unfolding process. The present two-state model provided the *m* values as a measure of the effectiveness of various carboxylic acids as previously reported [19]. The role of carboxylic acids on the conformational transition of protein (*m* values) was discussed, focusing on the additive contribution of each constituent group of the carboxylic acids using the accessible surface area (ASA) of the constituent groups, i.e., the hydrocarbon (CH) group, hydroxyl (OH) group, halogen substituents, and the carboxyl (COOH) group.

2. Experimental

2.1. Materials

The abbreviations for the carboxylic acids are summarized in Table 1. MeCOOH, EtCOOH, PrCOOH, *n*BuCOOH, *i*BuCOOH, ClEtCOOH, TFA, and PFPA were purchased from Wako (Osaka, Japan) and were used without further purification. Bovine β-lactoglobulin (β-LG), isomer A, was purchased from Sigma (St. Louis, MO, USA) and was used without further purification.

2.2. Preparation of solutions

The desired concentration of protein was incubated overnight in a pH 2 medium (20 mM HCl). The saturated protein solution was mixed into a 20 mM HCl/carboxylic acid mixture and was incubated for 3 h before measurement [28]. The pH was measured using a Radiometer PHM83 at 25 °C.

2.3. Fluorescence measurements

The fluorescence measurements were carried out using 1-cm^2 quartz cells in a Jasco FP-6500 spectrofluorometer fitted with a water-jacketed cell holder at 25 °C in a water bath. For the measurement of the tryptophan (Trp) fluorescence, the excitation wavelength was 290 nm and the emission wavelength was recorded from 300 to 450 nm [35]. Samples containing carboxylic acids at different concentrations were equilibrated for 3 h at 25 °C before recordings were made. The protein concentration was 10 μM for the Trp fluorescence measurements. Relative fluorescence changes were expressed as I_x/I_0, where I_x is the peak fluorescence intensity of β-LG in the presence of various concentrations of carboxylic acids, and I_0 is the initial value of fluorescence intensity in the absence of carboxylic acid. A background experiment was performed to determine the effects of HCl/carboxylic acid mixture in the absence of protein.

2.4. Two-state transition analysis

In order to analyze the effect of carboxylic acids on β-LG, a two-state transition between the native state (N) and the unfolded state (U) was assumed. The apparent equilibrium constant for unfolding, K_U, was defined by $K_U = [U]/[N]$, where [N] and [U] are the concentrations of the native and unfolded states, respectively. The K_U value was determined from the value of normalized fraction of unfolded β-LG (f_U), where K_U could be calculated as $f_U/(1 - f_U)$. The free energy change of the denaturation was calculated as follows: $\Delta G_U = -RT \ln K_U$, where R is the gas constant and T is the absolute temperature in Kelvin. The linear dependence of ΔG_U upon the concentration of carboxylic acid, [carboxylic acid], can thus be derived as follows based on Goto's