trans-cyclohexanediamines prevent thermal inactivation of protein: Role of hydrophobic and electrostatic interactions.

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Running title

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Abbreviations

RNase A, Ribonuclease A; CHDA, *trans*-cyclohexanediamine; 1,2-CHDA, *trans*-1,2-cyclohexanediamine; 1,4-CHDA, *trans*-1,4-cyclohexanediamine; Arg, Arginine; Gdn, Guanidine; Phe, Phenylalanine; Ile, Isoleucine; cCMP, Cytidine 2′:3′-cyclic monophosphate sodium salt; HPLC, High performance liquid

chromatography.

Abstract

Although solution additives prevent protein misfolding, the mechanism remains elusive. In this paper, we compare the preventive effects of trans-1,2-cyclohexanediamine and trans-1,4-cyclohexanediamine on the heat-induced inactivation of ribonuclease A and lysozyme. These additives are more effective in preventing thermal inactivation of the proteins than guanidine and arginine. The results suggest two possibilities: (i) decrease in the hydrophobic interaction between unfolded protein molecules is indispensable for preventing protein association, and (ii) the electrostatic interaction between additives interacting with the hydrophobic residues of protein molecules plays an important role in preventing thermal inactivation of proteins.

Keywords

Inactivation; lysozyme; ribonuclease A; hydrophobic interaction; electrostatic interaction

1. INTRODUCTION

Protein aggregation is a serious problem for biotechnology and pharmaceutical applications (Fink, 1998; Kopito, 2000; Tsumoto *et al*., 2003). Many studies have shown that the aggregation proceeds as a first-order reversible folding/unfolding reaction and subsequent intermolecular association with a higher-order irreversible process (Georgiou *et al*., 1994; Winzor *et al*., 1992). The aggregation is generally affected by the solution conditions, such as temperature, pH, concentration of protein, and the presence of additives. The additives affect the stability of the native structure and the aggregation rate of proteins (Tsumoto *et al*., 2004; Shiraki *et al*., 2004, Hirano *et al*., 2007). Guanidine (Gdn) and urea are well-used additives as aggregation suppressors that weaken hydrophobic intermolecular interaction of proteins (Dunbar *et al*., 1997; Bhuyan, 2002). These denaturant molecules increase the solubility of aggregation-prone unfolded molecules, but decrease the stability of the native state. Arginine (Arg) has been used in various techniques, such as chromatography (Ejima *et al*., 2005a, b), refolding (Tsumoto *et al*., 1998; Umetsu *et al*., 2003; Ishibashi *et al*., 2005; Chow *et al*., 2006; Ejima *et al*., 2006), and thermal aggregation suppression (Shiraki *et al*., 2002), because it does not destabilize the native state but increases the solubility of aggregation-prone molecules (Tsumoto *et al*., 2004;Arakawa and Tsumoto,

2003; Shiraki *et al*., 2002; Shiarki *et al*., 2004).

Many chemicals containing an amino group effectively prevent heat-induced aggregation and inactivation of lysozyme, such as polyamines (Kudou *et al*., 2003), diamines (Okanojo *et al*., 2005), amino acid derivatives (Shiraki *et al*., 2005; Hamada and Shiraki, 2007; Matsuoka *et al*., 2007), and ammonium ion (Hirano *et al*., 2007). Although these additives have been developed to control protein aggregation in the field of biotechnology, only a few molecular mechanisms have been elucidated, such as (i) monoamine and diol did not prevent thermal inactivation and aggregation of lysozyme (Okanojo *et al*., 2005); (ii) amine compounds show weak denaturing properties of proteins (Okanojo *et al*., 2005); (iii) ammonium ions increase the solubility of hydrophobic amino acids (Hirano *et al*., 2007).

Recent research on additives suggests that the solubility of hydrophobic amino acids is an important factor in assessing the effect of additives, because it indicates the strength of the interaction with hydrophobic residues of protein (Tsumoto *et al*., 2004; Hirano *et al*., 2007). Briefly, the additives that dissolve hydrophobic amino acids may be better at preventing thermal inactivation and aggregation due to weakened hydrophobic interaction between protein molecules.

In this paper, we show that trans-cyclohexanediamines (CHDAs) prevent thermal

inactivation of ribonuclease A and lysozyme and we propose two mechanisms for prevention of heat-induced inactivation of proteins.

2. MATERIALS AND METHODS

2.1. Materials

Hen egg white lysozyme, bovine ribonuclease A (RNase A), cytidine 2′:3′-cyclic monophosphate sodium salt (cCMP), Arg, Gdn, and 3-(N-morpholino)propanesulfonic acid were obtained from Sigma Chemical Co. (St. Louis, USA). NaH_2PO_4 was obtained from Nacalai Tesque, Inc (Kyoto, Japan). *trans*-1,2-cyclohexanediamine (1,2-CHDA) and trans-1,4-cyclohexanediamine (1,4-CHDA) were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). HCl, NaOH, and *Micrococcus lysodeikticus* were obtained from Wako Pure Chemical Indus., Ltd. (Osaka, Japan). All chemicals used were of high-quality analytical grade.

2.2 Residual activity of lysozyme after heat treatment

The bacteriolytic activity of lysozyme was estimated as follows. A total of 1.5 ml of 0.5 mg/ml *Micrococcus lysodeikticus* solution prepared in 50 mM sodium-phosphate buffer was adjusted to pH 7.0 by HCl or NaOH and was mixed with 10 μl of the protein

solution. The decrease in light scattering intensity of the solution was monitored by measuring the absorbance at 600 nm. The residual activity was estimated from the slope of the initial decrease in the intensity.

2.3. Residual activity of RNase A after heat treatment

The activity of RNase A was estimated as follows. A total of 1.5 ml of 0.1 mg/ml cCMP solution prepared in 0.1 M 3-(N-morpholino)propanesulfonic acid buffer was adjusted to pH 7.0 by HCl or NaOH and was mixed with 10 μl of the protein solution. The increase in the light scattering intensity of the solution was monitored by measuring the absorbance at 284 nm. The residual activity was estimated from the slope of the initial increase in the intensity.

2.4. Thermal inactivation in the presence of CHDAs

Heat-induced inactivation of RNase A and lysozyme were performed as follows. A stock solution containing 2.0 mg/ml RNase A or 0.5 mg/ml lysozyme, 50 mM Na-phosphate buffer, and 0-0.1 M CHDAs, Arg, or Gdn was prepared and adjusted to pHs 7.0 or 8.3 by HCl or NaOH. A total of 200 μl of the stock solutions were added to micro disposal tubes. The solutions were heated at 98°C for measured periods (10 min

or 3 min) using a temperature control system PC-880 (Astec, Fukuoka, Japan). After the heat treatment, the samples were centrifuged at 15,000 *g* for 20 min at 20°C, and then the amounts of residual activity of soluble protein were measured.

2.5. Time course of thermal inactivation

Heat-induced inactivation of RNase A and lysozyme was performed as follows. A stock solution containing 2.0 mg/ml RNase A or 0.5 mg/ml lysozyme, 50 mM Na-phosphate buffer, and 0.1 M CHDAs was prepared. A total of 200 μl of the stock solutions was added to micro disposal tubes. The solutions were heated at 98°C for various periods using a temperature control system PC-880. After the heat treatment, the samples were centrifuged at 15,000 *g* for 20 min at 20°C, and then the amounts of residual activity of soluble protein were measured. The obtained data were fitted to single exponential equations and the rate constants of inactivation were estimated. The all measurements were performed three times and the standard errors were plotted.

2.6. Solubility of amino acids in CHDA solutions

Solution containing 1.0 M CHDAs and excess concentrations of phenylalanine (Phe) or isoleucine (Ile) were adjusted to various pHs by HCl or NaOH and were incubated with

shaking at 37 °C for 1 hour. After that, the samples were incubated at 25°C for 10 hour. After the treatment, the samples were filtrated by 0.20 μm disposable syringe filter from Toyo Roshi Kaisha, Ltd. (Tokyo, Japan). The soluble concentrations of amino acids were determined by high performance liquid chromatography (HPLC). HPLC equipment consisted of BioLogic DuoFlow system (Bio-Rad Laboratories, Inc., Tokyo, Japan) and a reverse phase C30 column, Develosil RPAQUEOUS-AR-5 (Nomura Chemical Co, Ltd., Aichi, Japan). The solutions containing saturated amino acid and 0.1 M CHDAs were diluted 25-fold for Phe solution or 10-fold for Ile solutions with pure water and then the samples of 20 μl were injected in the HPLC. The mobile phases for analysis of Phe and Ile were pure water and 5% acetonitrile with 0.1% TFA in pure water, respectively. The flow-rate was 1.0 ml/min at room temperature. The solubility was determined by the peak areas and was calculated by the calibration curves. The all measurements were performed three times and the standard errors were plotted. The detection wavelengths of Phe and Ile were 220 and 205 nm, respectively.

3. RESULTS AND DISCUSSION

3.1 Thermal inactivation of RNase A and lysozyme in the presence of CHDAs

Figure 1A and B show the amounts of residual activity of 2.0 mg/ml RNase A in the

presence CHDAs, Arg, or Gdn after heat treatment at 98°C for 10 min (pH 7.0) and 3 min (pH 8.3). The residual activity of RNase A increased with increasing concentration of CHDAs. At pH 7.0, the preventive effect of 1,2-CHDA on the heat inactivation was higher than that of 1,4-CHDA at pH 7.0 (Fig. 1A), while at pH 8.3, the preventive effect of 1,4-CHDA was higher than that of 1,2-CHDA (Fig. 1B). Figure 1C and D show residual activity of 0.5 mg/ml lysozyme in the presence of CHDAs, Arg, or Gdn after the heat treatment at 98°C for 10 min (pH 7.0) and 3 min (pH 8.3). The data for lysozyme were similar to those for RNase A; the residual activity of lysozyme increased with increasing concentration of CHDAs. CHDAs had a more favorable effect than Arg and Gdn for preventing the thermal inactivation of the proteins.

Figure 2 shows time course of the thermal inactivation of 2.0 mg/ml RNase A and 0.5 mg/ml lysozyme in the presence of 0.1 M CHDAs at pH 7.0 and 8.3. At pH 7.0, the thermal inactivation rates of RNase A and lysozyme in the presence of 1,2-CHDA were slower than in the presence of 1,4-CHDA (Fig. 2A and C). However, at pH 8.3, the inactivation rates of each protein in the presence of 1,4-CHDA were slower than that in the presence of 1,2-CHDA (Fig. 2B and D). These data indicate that the ability of CHDAs to prevent the thermal inactivation of the proteins depends on the pHs; 1,2-CHDA was favorable at pH 7.0, while 1,4-CHDA was favorable at pH 8.3.

Figure 3 shows the relationships between inactivation rate constants and the pH for RNase A and lysozyme. The rate constants of the inactivation for each protein in the presence of 1,2-CHDA were lower than in the presence of 1,4-CHDA below pH 7.8. However, above pH 7.8, the rate constants in the presence of 1,4-CHDA were lower than those of 1,2-CHDA. The rate constants of the inactivation in each CHDA solution were equal at around pH 7.8.

3.2. Solubility of hydrophobic amino acids in the presence of CHDAs

Additives that prevent protein inactivation and aggregation increase the solubility of hydrophobic amino acids (Hirano *et al*., 2007). In order to clarify the effect of CHDAs in preventing the inactivation, we investigated the solubility of hydrophobic amino acids (Phe and Ile) in the presence of 1,2-CHDA and 1,4-CHDA at various pHs (Fig. 4). At neutral pHs, Phe and Ile in 1 M 1,2-CHDA solution were dissolved more than in 1,4-CHDA. At alkaline pHs, however, 1,2-CHDAs dissolved the hydrophobic amino acids as much as 1,4-CHDA.

3.3. Effects of CHDAs for preventing thermal inactivation of proteins

The data for the thermal inactivation of the proteins with CHDAs are summarized as

follows: (i) below pH 7.8, 1,2-CHDA prevented the thermal inactivation of the proteins more effectively than 1,4-CHDA; (ii) above pH 7.8, 1,4-CHDA prevented the thermal inactivation of the proteins more effectively than 1,2-CHDA; (iii) below pH 8.5, 1,2-CHDA showed higher solubility of hydrophobic amino acids than 1,4-CHDA; (iv) above pH 8.5, 1,2-CHDA and 1,4-CHDA showed similar solubility of the hydrophobic amino acids.

Consideration of the solubility of amino acids partially accounts for the thermal inactivation of the proteins in the presence of CHDAs. Since 1,2-CHDA dissolve more hydrophobic amino acids than 1,4-CHDA below pH 7.8, 1,2-CHDA molecules may interact with the hydrophobic surface of thermal unfolded protein molecules more favorably than 1,4-CHDA, leading to increased solubility of aggregation-prone misfolded molecules and more effective prevention of thermal inactivation than 1,4CHDA below pH 7.8. On the other hand, 1,4-CHDA more effectively prevented the protein inactivation than 1,2-CHDA in the alkaline pH region (>7.8) (Fig. 3), although 1,2-CHDA dissolved hydrophobic amino acids not less than 1,4-CHDA in the pH region (Fig. 4). Therefore, not all of the solubility of the hydrophobic amino acids in CHDA solutions explains the prevention of the thermal inactivation of the proteins.

As CHDAs have two possible protonation sites, it is important to know the charged

states of CHDAs to understand the ability of the additives to prevent the thermal inactivation of proteins. Figure 5 shows pH titration profiles of 1,2-CHDA and 1,4-CHDA. The pK_a of 1,2-CHDA were at around 6.5 and 10, while those of 1,4-CHDA were at around 10. In acidic pH (<4.5), a net charge of 1,2-CHDA was about 2.0, which was similar to that of 1,4-CHDA, whereas in pH 5-11 (especially, pH 7.8-8.5), a net charge of 1,4-CHDA was larger than that of 1,2-CHDA. At pH 7.8, the net charge of 1,4-CHDA was about 2.0, while that of 1,2-CHDA was about 1.0. At pH 8.5, the charge of 1,4-CHDA was about 1.9, while that of 1,2-CHDA was about 1.0.

This fact supports the hypothesis that 1,4-CHDA would electrostatically prevent protein inactivation above pH 7.8 even if 1,4-CHDA interacts with hydrophobic residues of the protein less than 1,2-CHDA in the pH region (Fig. 4).

In this work, new additives CHDAs that prevent thermal inactivation were found and two mechanisms were proposed; these data suggest that the prevention of the thermal inactivation depends on not only the hydrophobic interaction between hydrophobic surfaces of protein molecules but also the electrostatic interaction between CHDA molecules interacting with protein molecules. These findings are useful for biotechnology and pharmaceutical industries.

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FIGURE CAPTIONS

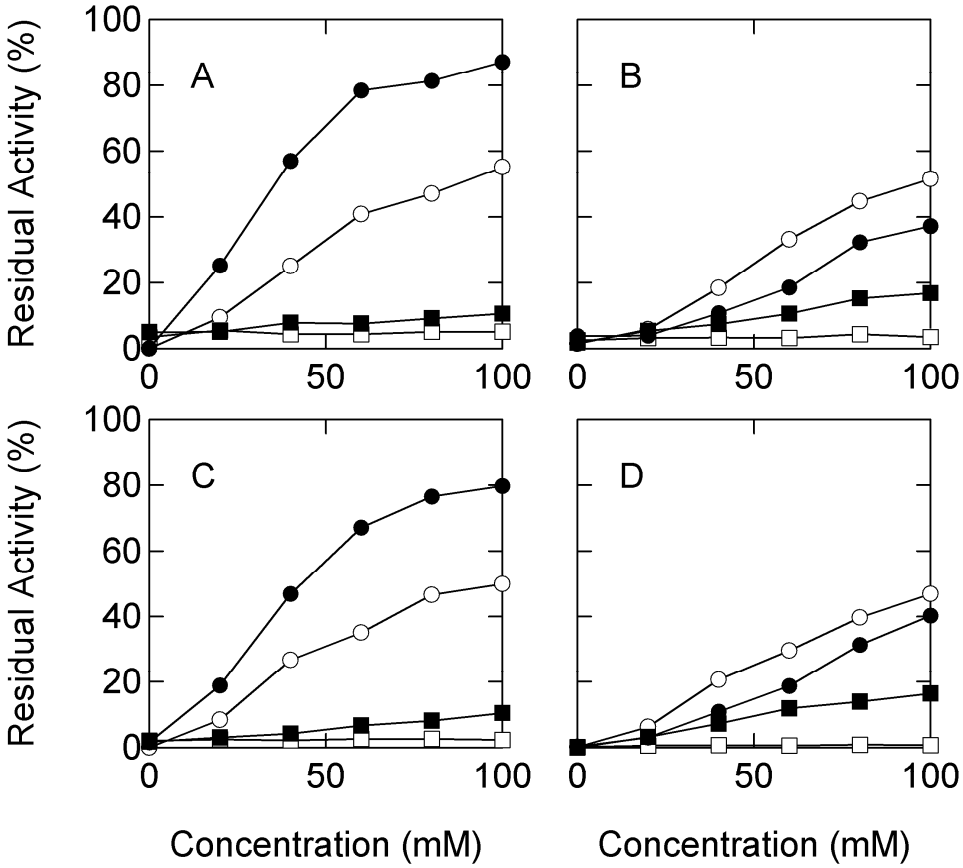
Fig. 1. Heat-induced inactivation on RNase A and lysozyme in the presence of CHDAs. Samples containing 2.0 mg/ml RNase A (A,B) and 0.5 mg/ml lysozyme (C,D) in the presence of CHDAs, Arg, or Gdn at pH 7.0 (A,C) and pH 8.3 (B,D) were heated at 98°C for 10 min (A,C) or 3min (B,D). 1,2-CHDA, closed circles; 1,4-CHDA, open circles; Arg, closed squares; Gdn, open squares.

Fig. 2. Representative data for heat-induced inactivation of RNase A and lysozyme in the presence of additives. Samples containing 2.0 mg/ml RNase A (A,B) or 0.5 mg/ml lysozyme (C,D), 50 mM Na-phosphate, and 100 mM additives were heated at 98°C for the respective periods at pH 7.0 (A,C) and at pH 8.3 (B,C). After the heat treatment, the residual activity was determined. 1,2-CHDA, closed circles; 1,4-CHDA, open circles. The continuous lines show the theoretical curves fitted to the data with single exponential equations.

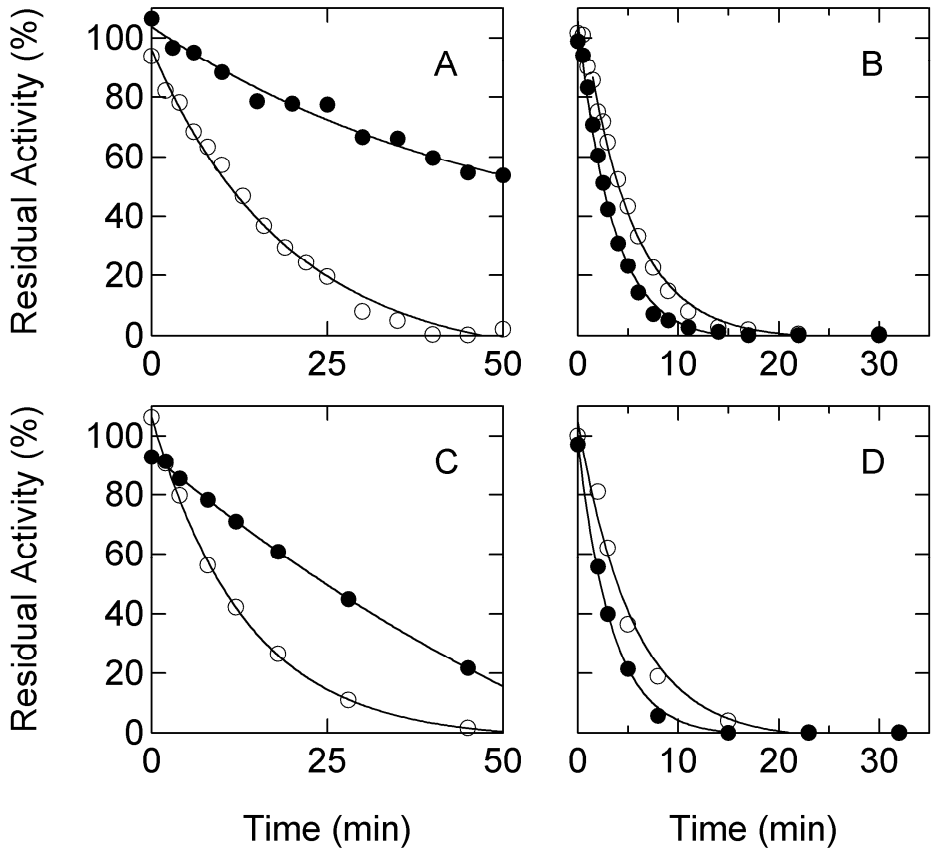
Fig. 3. Rate constants of heat-induced inactivation of RNase A (A) and lysozyme (B) at various pHs. 1,2-CHDA, closed circles; 1,4-CHDA, open circles.

Fig. 4. The solubility of Phe (A) and Ile (B) in the presence of 1.0 M CHDAs at various pHs. 1,2-CHDA, closed circles; 1,4-CHDA, open circles.

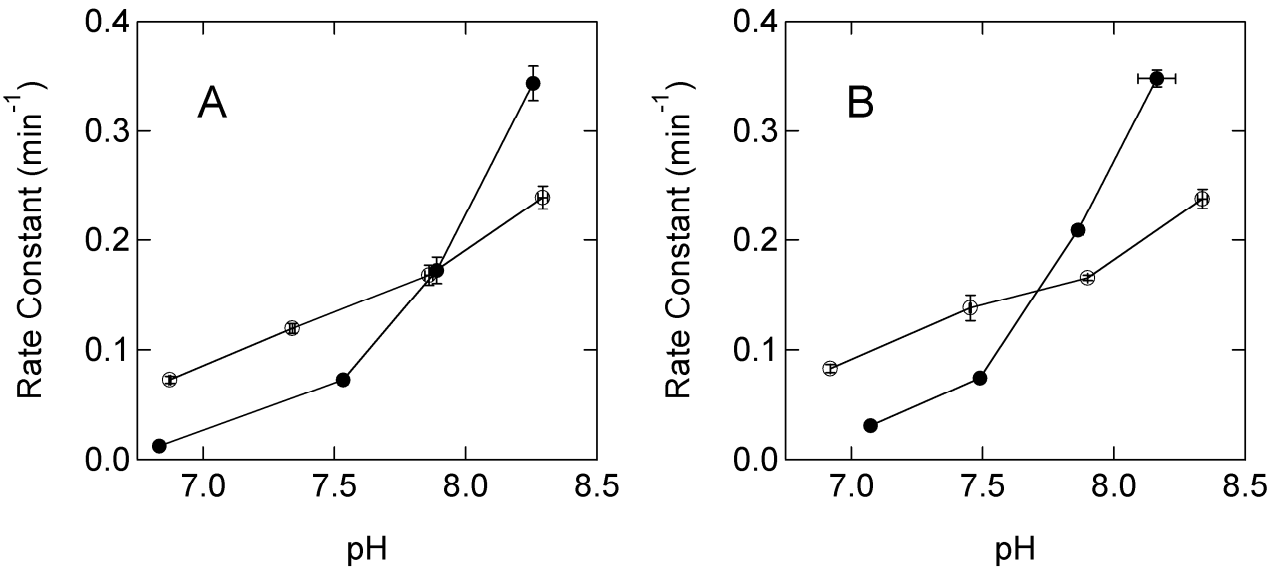
Fig. 5. Estimation of net charge of 1,2-CHDA and 1,4-CHDA at various pHs by the titration. A small quantity of 1.0 M HCl was added to 5 mL of 50 mM 1,2-CHDA (closed circles) or 1,4-CHDA (open circles) solution.



Hirano et al., Fig. 1



Hirano et al., Fig. 2


A
B
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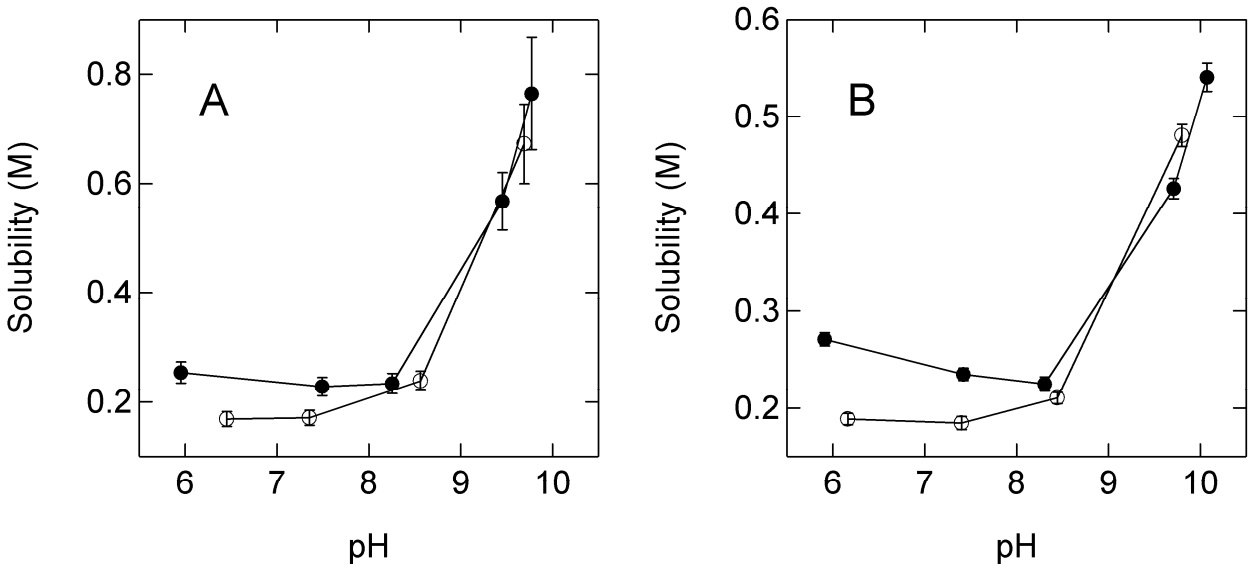
8.0
8.5


A
B
Solubility (M)
pH
0.2
0.4
0.6
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Hirano et al., Fig. 5