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Solvent-free crystallizations of amino acids: The effects of the hydrophilicity/hydrophobicity of side-chains

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ABSTRACT

This work studied the self-assembling (crystallizing) behaviour of amino acids in the absence of solvent and additives (by sublimation and deposition in vacuum), instead of from aqueous solution. It is found that the hydrophilicity/hydrophobicity of side-chains can significantly affect the crystallization of amino acids in the absence of solvent. Crystal structures of amino acids having hydrophobic side-chains (L-valine, L-leucine, L-isoleucine and L-methionine) obtained from sublimation are the same with those obtained from aqueous solution. New polymorphs for six amino acids are thought to have been obtained, based on X-ray diffraction and IR data for three of them (L-tyrosine, L-Phyenylalanine and L-tryptophan), and just IR data for the other three (L-alanine, L-proline and L-threonine).

1. Introduction

As the elementary basis of peptides and proteins, the molecules of amino acids have commanded our interest and attention. Crystalline amino acids are a link between chemistry, materials science and biology [1]. The studies of polymorphism of amino acids and the control of their structures and properties are important for practical applications of these compounds as non-linear optical materials, piezoelectrics and drugs [2–4]. The three-dimensional crystalline structures of amino acids also can serve as biomimetics modeling interactions in biopolymers [1,5]. Phase transition in crystalline amino acids is related to the protein folding, because both the two things are related to the self-assembling of amino acid molecules [6,7]. At relatively modest pressures proteins tend to unfold, water being forced into their interior. Under different hydration condition or additives, different folding modes of proteins can be formed [8]. To date, it is unclear how the environment affects the interactions of amino acid composition in proteins. As a prelude to answering this question, it is absolutely necessary to examine the effects of crystallizing condition on the individual amino acids.

Generally, amino acids were crystallized from aqueous solutions. Crystal structures of amino acids with hydrophobic side-chains (L-valine, L-leucine, L-isoleucine and L-methionine), are always divided into distinct double-layers (hydrophilic and hydrophobic layers) [9,10]. Most of amino acids with hydrophilic side-chains form single layer arrangements in crystals [1]. Each crystal structure in the hydrophilic group (including polar, acidic and basic amino acids) has its own unique packing arrangement that not only the amino and carboxyl groups, but also donating and accepting groups in the side-chain can involve in hydrogen-bonding (H-bonding) networks [11].

The crystallization is a not only thermodynamic but also kinetically determined process, and the precipitation of various polymorphs can be induced at different conditions, such as different solvents or additives [12,13]. Here we explore the self-assembling (crystallizing) behaviour of amino acids in the absence of solvent and additives (by heating and sublimation), instead of from aqueous solution. We found that the hydrophilicity/hydrophobicity of side-chains can significantly affect the crystallization of amino acids in the absence of solvent. Crystal structures of amino acids having hydrophobic side-chains (L-valine, L-leucine, L-isoleucine and L-methionine) obtained from sublimation are the same with those obtained from aqueous solution. New polymorphs for six amino acids are thought to have been obtained, based on X-ray diffraction and IR data for three of them (L-tyrosine, L-Phyenylalanine and L-tryptophan), and just IR data for the other three (L-alanine, L-proline and L-threonine).

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2. Materials and methods

2.1. Chemicals

The amino acids (anhydrous forms) were commercially obtained from Sigma-Aldrich and Sinopharm Chemical Reagent. The H_2O was doubly distilled. H_2O was further purified by freeze–thaw cycles in the vacuum system.

2.2. Apparatus and methods

The sublimation of amino acids in vacuum and its polymorphic transformation were studied using X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) techniques. In this work, amino acids were rapidly heated and sublimated in vacuum (300 °C, <0.1 Pa) and deposited spontaneously onto a CaF_2 wafer in a quartz cell (the diagram of the cell is shown in Supplementary Material Scheme S1). The quartz cell used has a 15mm, gap distance between the CaF_2 wafer and the bottom where the amino acids were placed and heated to sublimation. The quartz cell was vacuumed by a pump (to 0.1Pa) for 30min and then closed before sublimation.

As the sublimates exposed to ambient condition, their XRD patterns were measured in 30min. The powders of the sublimates and known form of amino acids were ground before the XRD examination to avoid the effect of preferred orientation on diffraction patterns. The powder X-ray diffraction patterns were collected on an X-ray diffractometer (Rigaku D/Max2500VB2). XRD experimental parameters: Cu Kα (0.15418nm, 40kV/100mA), 5°/min, 2–70°, reflection geometry.

In order to examine the states of the sublimates in vacuum, the sublimates of amino acids were examined by infrared (IR) spectroscopy equipped with a specially fabricated in-situ IR cell in which the sublimation and IR measurement can be conducted simultaneously under vacuum (the diagram of the IR cell is shown in Scheme S1). All the IR spectra were collected on a FTIR spectrometer (Nicolet Nexus 470) with a resolution of 4 cm^{-1} and 64 scans in the region of 4000–1000 cm^{-1}.

The elements analysis was done on a CHN element analyzer (Perkin-Elmer 2400 IT). The weight percentage of oxygen is obtained by: O %=100% − (C %+H %+N %).

3. Results

3.1. XRD characterization of the sublimates of amino acids

Some amino acids, such as L-alanine, L-methionine and L-tryptophan don't decompose (recovery higher than 99%) and retain their optical rotation when subjected to the sublimation procedure in vacuum [14]. White fine powders were obtained for these amino acids after sublimation in vacuum. To verify whether the sublimates of these amino acids exist in ordered structures or amorphous states, the sublimates were examined by powder X-ray diffraction (PXRD) firstly. The collected XRD data of sublimates were compared with both the previously published powder XRD data and the experimental XRD data of the starting materials. XRD data of the starting materials are consistent well with the published data, which means that the starting materials exist as the known phases.

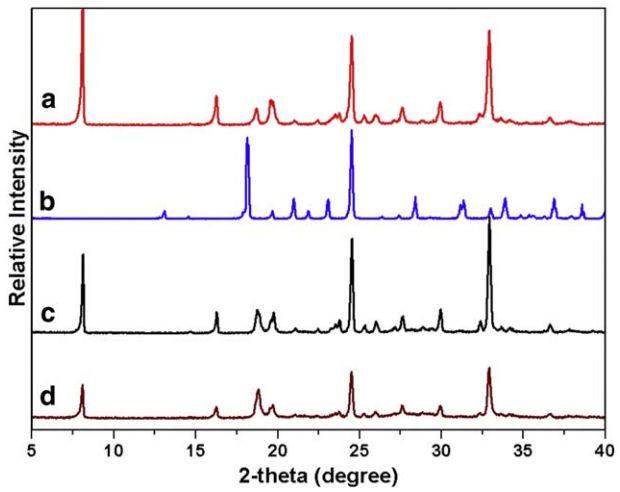


Fig. 1. XRD of: (a) the sublimate of L-cysteine, (b) pattern consistent with L-cysteine-I, (c) pattern consistent with L-cysteine-II and (d) the powder of residual L-cysteine-I which does not sublime during heating in vacuum.

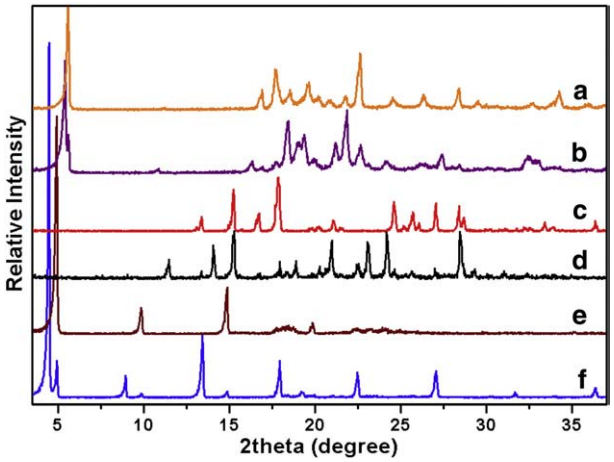


Fig. 2. XRD of: (a) the known form of L-phenylalanine, (b) the sublimate of L-phenylalanine, (c) the known form of L-tyrosine, (d) the sublimate of L-tyrosine, (e) the known form of L-tryptophan and (f) the sublimate of L-tryptophan.

L-cysteine was known to crystallize in two polymorphs, an orthorhombic phase (CCDC No. 282711, $P2_12_12_1$, $Z=1$) [15] and a monoclinic phase (CCDC No. 126977, $P2_1$, $Z=2$) [16]. We refer to these phases as L-cysteine-I and L-cysteine-II, respectively. The two forms of L-cysteine can display different XRD patterns and IR spectra due to the different stacking and H-bonding networks. The powder XRD patterns (Fig. 1) showed that the sublimates of L-cysteine-I and -II exist as the crystalline state of L-cysteine-II. These results indicate that L-cysteine-II is the preferential polymorph when L-cysteine crystallized from gas phase. XRD characterization also reveals that a solid transformation of L-cysteine-I to L-cysteine-II occurred before sublimation during heating. Glycine has similar situation to L-cysteine in some degree. In our previous study about the crystallization of glycine, we have found that β glycine can be crystallized from gas phase via the sublimation of α or γ form in vacuum [17].

Interestingly, the sublimates of L-tyrosine, L-phenylalanine and L-tryptophan (Fig. 2) show new XRD patterns in addition to those of their known crystal forms respectively. If peptides or anhydride were formed in the sublimation process, the percentages of elements would change remarkably. Elemental analysis of L-phenylalanine, L-tryptophan and their sublimates (in Supplementary material Table S2) makes sure that no decomposition and condensation of the amino acids occur during the sublimation and are not the reasons for the generation of the unprecedented XRD patterns via sublimation. It is clear that new polymorphs of these three amino acids are formed after the sublimation in vacuum. The new polymorph of L-tryptophan is crystallized in the form of tiny strips, which is different from the platelets of its known form, as showed by the photomicrographs (in Supplementary material in Fig. S1).

Unfortunately, it has not been possible to grow single crystals of the new metastable polymorphs for X-ray structure determination. An initial indexing from the new powder XRD peaks in Fig. 2f (compared with Fig. 2e) suggests an orthorhombic unit cell of the new polymorph of L-tryptophan with lattice parameters: $a=19.771$ Å, $b=10.363$ Å,

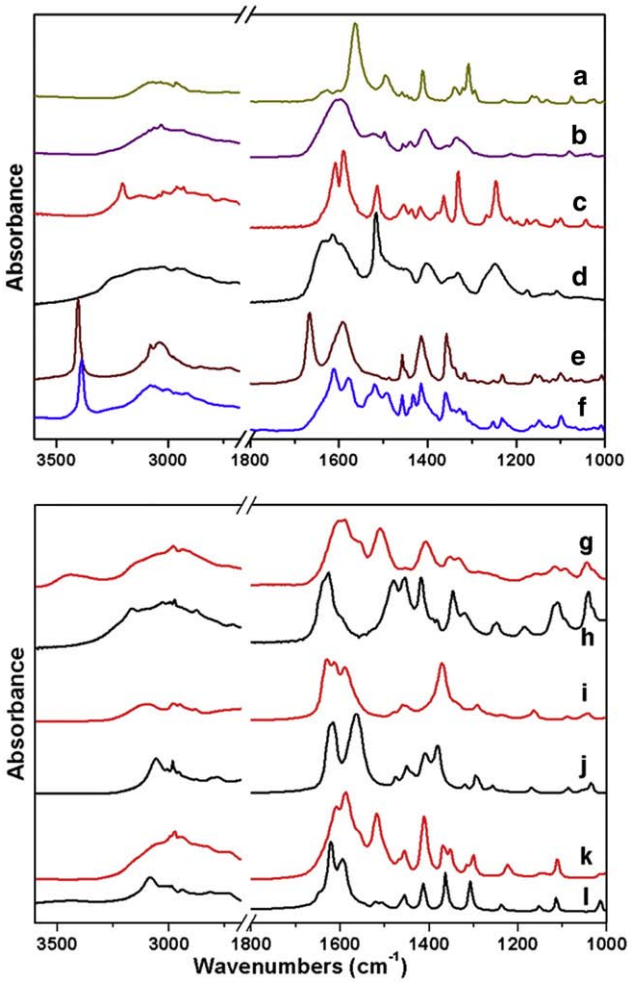


Fig. 3. The IR spectra of: (a) the known form of L-phenylalanine, (b) the sublimate of L-phenylalanine, (c) the known form of L-tyrosine, (d) the sublimate of L-tyrosine, (e) the known form of L-tryptophan, (f) the sublimate of L-tryptophan, (g) the sublimate of L-threonine, (h) the known form of L-threonine, (i) the sublimate of L-proline, (j) the known form of L-proline, (k) the sublimate of L-alanine and (l) the known form of L-alanine.

c=9.962 Å, which is different from the known crystalline form of L-tryptophan (orthorhombic, 16.81 * 17.9 * 6.904) [18].

The sublimates of the other amino acids (L-isoleucine, L- valine, L-methionine, L-proline, L-alanine, L-leucine, and L-threonine) show the same XRD patterns with their known forms respectively. Consistent with Gross's report [14], L-serine, L-lysine and L-glutamic acid decompose when subjected to the sublimation procedure in vacuum, as indicated by the blackening of the material and some liquid emerged in the sublimation apparatus.

3.2. IR spectra of the sublimates of amino acids in vacuum

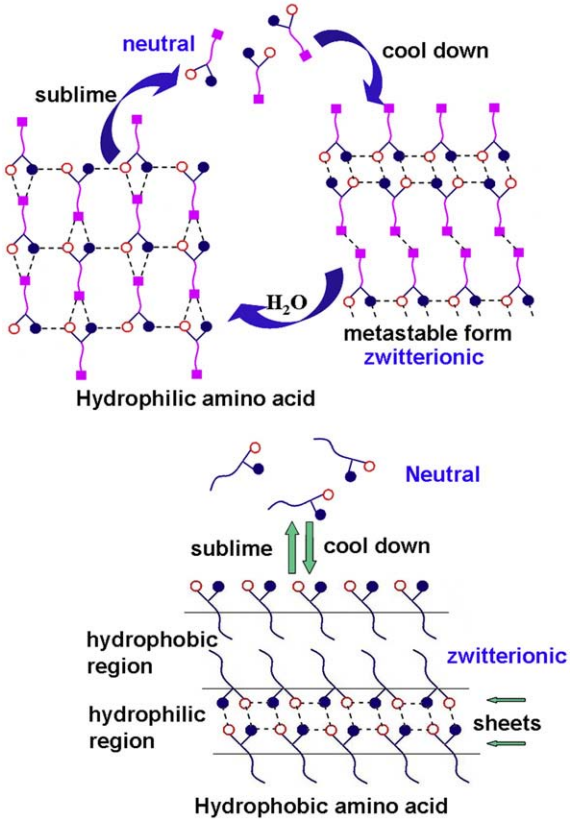
IR spectroscopy is a simple and reliable tool for the investigation of polymorphism [19] and is also convenient to study the crystalline form of amino acids in vacuum. Different polymorphs of the same molecules can result in changes in IR band positions, band shapes, and absence or presence of a few bands. This may be enough to characterize a whole series of polymorphs, for example, all nine polymorphs and solvates of phenobarbitone were clearly distinguishable by IR spectroscopy [20]. The sublimates of hydrophobic amino acids (L-leucine, L-isoleucine, L-valine and L-methionine) show the same XRD patterns and IR spectra with their known forms respectively, which means that the sublimates of hydrophobic amino acids have the same crystalline forms with their known forms.

Table 1
Crystalline form of amino acids after sublimation and deposition in vacuum

Metastable form (Class I)		Still regular form (Class II)	Decomposition (Class III)
New polymorphs	Known polymorphs		
L-tryptophan	Glycine	L-valine	L-serine
L-tyrosine	L-cysteine	L-leucine	L-lysine
L-phenylalanine		L-isoleucine	L-glutamic acid
L-alanine		L-methionine	
L-proline			
L-threonine			

Surprisingly, not only L-phenylalanine, L-tyrosine and L-tryptophan, but also L-alanine, L-proline and L-threonine (Fig. 3), showed totally different IR spectra of their sublimates from those of their known forms respectively. These novel IR spectra have never been reported for the solids of these amino acids before. Amino acids are not present as neutral forms but as zwitterions in the sublimates because there are no IR bands in the range of 1700–1800 cm^{-1} (C=O stretching vibrations) [21,22]. In all the spectra there are broad intense bands from 2200 to 3400 cm^{-1} due to the N–H stretch of NH_3^+ hydrogen bonded with COO^- in the crystal lattice. This absorption showed in all the IR spectra of amino acid solids means that the strong H-bonding networks are present in the large multimers (tiny crystals) of the known form solids and sublimates [23,24].

IR spectra also showed that the sublimates of these six amino acids in Fig. 3 are stable in vacuum and can transform back their known forms with different speeds after being exposed to air. This transformation for L-threonine was shown in Fig. S3 (see Supplementary material) as an example. It is proved that it is not N_2 and O_2, but water vapor in air inducing the transformation from metastable into stable forms. After the transformation, the amino acids keep its known



Scheme 1. The phase transformations of hydrophobic (bottom) and hydrophilic (top) amino acids during sublimation. The carboxyl, amino groups and hydrophilic side-chains are represented by hollow balls, solid balls and squares respectively.

IR characters even the inducers (water vapor) are evacuated from the IR cell. Apparently, metastable polymorphs of L-alanine, L-proline and L-threonine are also formed via sublimation. But the XRD patterns of their metastable polymorphs are hard to get due to the low stabilities of the new phases and irreversible transformations into their stable polymorphs in short time (less than 20 min) under ambient condition. The mechanism of solution-mediated phase transformation may be right for the transformation of metastable polymorphs of these amino acids into their stable forms [25]. L-tyrosine, L-phenylalanine and L-tryptophan have relatively longer natural lives of their metastable polymorphs under ambient condition and lower solubility in H_2O than L-alanine, L-proline and L-threonine.

4. Discussion

Bernal et al. had shown that the polymorphs of α-amino acids can be resulted from not only the changes in molecular conformations but also the changes in the arrangement patterns (without changes in molecular conformation) [26]. From the IR and XRD results, it is hard to determine whether the conformations of amino acids in polymorphs are same or different. But the H-bonding arrangements between amino acids molecules are different in polymorphs, as indicated by the changes of the IR spectra (1700–1500 cm^{-1}: asymmetric stretching of COO^- and asymmetric bending of NH_3^+; 2800–3600 cm^{-1}: asymmetric stretching of NH_3^+ and other O–H or N–H groups) [27]. In the crystals of L-tryptophan [18] and its sublimates, the NH group of the indol ring does not form the hydrogen bond with other groups, so that sharp IR bands at 3404 cm^{-1} (Fig. 3e) and 3388 cm^{-1} emerge in the IR spectra of its known form and sublimate (Fig. 3f) respectively. The bands due to υ_{OH} are not observed in the high-frequency region of IR spectrum of known form of L-threonine (Fig. 3h), because OH group involves in the H-bonding network of L-threonine crystal [22]. A weak broad band emerges at ca. 3450 cm^{-1} in the IR spectrum of its sublimate (Fig. 3g), which indicates that OH group becomes weakly hydrogen bonded with COO^- or other OH.

The metastable polymorphs of the amino acids in class I (Table 1) can be formed via sublimation as evidenced by the XRD and IR results. In the cases of glycine [17] and L-cysteine, the XRD and IR results clearly show that the sublimate is one single, pure phase (known metastable phase). So, we deduce that the sublimates of amino acids of Class I in our study exist as pure phases in vacuum. After a careful examination of previous reports of polymorphism in these amino acids [18,28,29], it is concluded that the new metastable polymorphs of six amino acids (anhydrous form), including L-tryptophan, L-tyrosine, L-phenylalanine, L-alanine, L-threonine and L-proline, were observed for the first time. Consistent with Gross's report, L-serine, L-lysine and L-glutamic acid (class III) decompose when subjected to the sublimation procedure in vacuum.

α-amino acids could be classified as either hydrophobic or hydrophilic types depending on the side-chain functional types. All the four amino acids in class II of this study, are hydrophobic ones [9,30]. Crystal structures of hydrophobic amino acids, however, are always divided into distinct hydrophilic and hydrophobic layers (as shown in Scheme 1). When hydrophobic amino acids were crystallized from either aqueous solution or gas phase, the amino group interacts with the carboxyl group by H-bonding forces, and the hydrophobic side-chains of amino acids are inclined to assemble together by van der Waals forces [31], so that the double-layers structure is the only result due to the two types of forces mentioned above (Scheme 1). This phenomenon is consistent with "hydrophobic collapse" in protein folding in nature [32,33].

Most of the amino acids in class I are hydrophilic amino acids. When crystallized from aqueous solution, each crystal structure in the hydrophilic group has its own unique hydrogen-bonding arrangements that involve not only the amino and carboxyl groups, but also donating and accepting groups in the side-chain [11]. Abundant polymorphism may be formed in some systems with multiple H-bonding opportunities under different crystallizing conditions, such as solvents, additives, solute concentration, temperature, crystallizing speed and so on [12,13]. In this work, compared with general crystallization from aqueous solution, crystallization via sublimation offers a special condition in which only solute–solute interactions exist [34], and hydrophilic amino acids have the opportunity to form different H-bonding networks (polymorphs) from that obtained from aqueous solution (as suggested in Scheme 1). According to Ostwald's "Rule of Stages" [35], neutral species of amino acid (stable in gas phase, but unstable with the highest free energy in solid phase) does not transform directly to the most stable packing configuration of zwitterions, but prefers to the metastable polymorph (intermediate stages).

It is important to differentiate the interactions among amino acid units in protein from those between amino acid units and solvent (water), because both hydrophobic and hydrophilic associations exist in the life of organisms [36,37]. Whenever hydrophobic amino acids are crystallized in aqueous solution or in the absence of solvent, the double-layer structure are the preferential assembling patterns due to the H-bonding and hydrophobic interactions. Hydrophilic amino acids, which have multiple hydrogen-bonding opportunities, can form different assembling patterns depending on conditions around. This work clearly shows that solvent (water) has different effects on the self-assembling of hydrophobic amino acids and hydrophilic groups. Undoubtedly, this result has wider implications in intermolecular interactions, materials and protein folding [36,37].

5. Conclusions

Our research reveals that the hydrophilicity/hydrophobicity of side-chains can significantly affect the solvent-free crystallization of amino acids (sublimation in vacuum). Amino acids with hydrophobic side-chains (L-valine, L-leucine, L-isoleucine and L-methionine) are crystallized to the same structures, whenever they are crystallized in aqueous solution or via sublimation. Hydrophilic amino acids, which have multiple hydrogen-bonding opportunities, can form different assembling patterns by aqueous solution crystallization and sublimation crystallization. New polymorphs for six amino acids are obtained for the first time under the solvent-free (sublimation) crystallizing conditions, based on X-ray diffraction and IR data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2008.09.011.

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