

Protein Biochemistry, Synthesis, Structure and Cellular Functions

Alanine

Dietary Sources, Physiological Functions
and Health Benefits

Nova
Biomedical



Franco Olivares
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Editors

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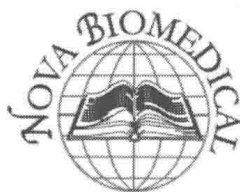
PROTEIN BIOCHEMISTRY, SYNTHESIS, STRUCTURE AND
CELLULAR FUNCTIONS

ALANINE

DIETARY SOURCES,
PHYSIOLOGICAL FUNCTIONS AND
HEALTH BENEFITS



MARTIN GOMEZ
EDITORS



New York

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PREFACE

The amino acid alanine is a nonessential amino acid. It can be synthesized in the human body and used to synthesize many different proteins. Alanine is found in a wide variety of foods, but is found in meats at higher concentrations. In this book, the authors present current research in the study of the dietary sources, physiological functions and health benefits of alanine. Topics discussed include α -alanine and calcium carbonates interaction for bio-mineralization; alanine and its role in bacterial spore biology; and the functions of alanine, beta-alanine and carnosine.

Chapter 1 - Bio-mineralization is the name given to a family of chemical and physical processes used by living beings to realize hard skeletons, shells, teeth and so on, which involve the reactions between organic and inorganic substances. Calcium carbonates are the base of most bio-mineral structures and \pm -alanine is known as a possible modifier for calcium carbonates crystallization phenomena, in which relative stability of the three CaCO_3 polymorphs is reversed. In order to provide a possible answer about which mechanisms could be involved with that, in this chapter, \pm -alanine crystallographic structure will be discussed, in order to predict equilibrium and growth shapes of related crystals. A short commentary about calcium carbonates chemistry and crystallography will be proposed and a brief summary about crystalline growth theories and computational crystallography will be provided. Techniques used for growing real crystals will be explained and obtained results will be proposed. Crystallization experiments, performed both in pure and calcium carbonate contaminated environment, will be compared, in order to show how the two substances, an organic one and an inorganic one, could interact.

Chapter 2 - The amino acid alanine has a role in nearly every major aspect of bacterial spore biology. This relationship exists in both the *Bacilli* and *Clostridia*, and no other amino acid appears to be as critical for spore success. The best understood interaction exists with mature spores that use germinant receptors to constantly probe their surroundings for germinants that signal and stimulate the major transition of germination back into a vegetative cell. Not only is L-alanine a germinant, but it is the only germinant that can solely trigger germination for nearly every investigated species of *Bacillus* and *Clostridium*. It is the primary indicator for spores that reproductive life is possible, and thus control of alanine is critical. In *Bacillus*, control is partially moderated by alanine-racemase (Alr) that isomerizes L-alanine to its stereoisomer D-alanine; a competitive inhibitor for germination. Alr is present in the outermost layers of the spore, will interact with L-alanine before it can penetrate to the germinant receptors, converts a significant amount of L-alanine to D-alanine, and thus dampens the germination response. This has been shown to be critical for *B. anthracis* spores that can delay germination in a mammalian host until it has penetrated the immune system, and then quickly shift out of spore-form to initiate disease. Yet, Alr starts using alanine to control spore success much earlier during the spore life cycle. During early sporulation, an excess of L-alanine is present and threatens to prematurely trigger germination of the developing forespore. That would mean death for the spore, but bimodal expression of Alr generates enough D-alanine to make sporulation a reliably safe venture for the bacteria.

Highly resistant spores have been a challenge to microbiologists who for decades have generated several highly toxic methods for killing spores. These brute force methods are variable in their effectiveness and fraught with public safety concerns, but our improved understanding of the alanine-spore interaction may someday change that. The crux of these new more “environmentally friendly” methods is to exploit the germination response to L-alanine to prematurely germinate spores and make them susceptible to milder decontaminants. Supplementing drinking water with L-alanine has been extensively tested by the US government to facilitate prevention of water-borne disease and as a countermeasure to bioterrorism. More recently, the role of Alr has been examined in hopes of possibly inhibiting its function. Inhibitors of Alr prevent D-alanine production and result in spores that are especially sensitive to L-alanine germination. One candidate inhibitor is D-cycloserine. It is capable of improving spore germination responses and potentially starts killing the germinating spore/outgrowing cell by interfering with cell wall synthesis before other gentle decontamination methods are later

applied. It is clear that control of alanine is of vital importance not only for humans trying to manipulate it to prevent disease, but also for the spore-forming bacteria that use it as a signal to indicate when its lineage can continue.

Chapter 3 - The alanine cycle depends on hepatic gluconeogenesis followed by glucose delivery and peripheral tissue utilization. Carnosine (beta-alanyl-L-histidine), a cytoplasmic dipeptide found at elevated concentrations in skeletal muscle, is formed from histidine and beta-alanine in a reaction catalysed by carnosine synthase. Carnosine is important in intracellular pH buffering over the physiological pH range. beta-alanine availability is limiting to carnosine synthesis in skeletal muscle. Hence, dietary intake of carnosine, or dipeptides that yield beta-alanine on absorption, or beta-alanine supplementation could augment intracellular buffering capacity during exercise, and thus enhance high-intensity exercise capacity and performance. Muscle carnosine serves as a physiological buffer, exhibits antioxidant properties, and affects enzyme regulation and sarcoplasmic reticulum calcium regulation. β -ALA supplementation increases skeletal muscle carnosine concentrations. Short-duration beta-alanine supplementation increases training volume and reduces subjective feelings of fatigue in college football players. Beta-alanine supplementation can significantly improve muscular endurance during resistance training in experienced resistance-trained athletes.

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Chapter 1 **α -ALANINE AND CALCIUM CARBONATES:
INTERACTION FOR BIO-MINERALIZATION***Francesco Massimino***ABSTRACT**

Bio-mineralization is the name given to a family of chemical and physical processes used by living beings to realize hard skeletons, shells, teeth and so on, which involve the reactions between organic and inorganic substances. Calcium carbonates are the base of most bio-mineral structures and $\hat{\text{I}}\pm$ -alanine is known as a possible modifier for calcium carbonates crystallization phenomena, in which relative stability of the three CaCO_3 polymorphs is reversed. In order to provide a possible answer about which mechanisms could be involved with that, in this chapter, $\hat{\text{I}}\pm$ -alanine crystallographic structure will be discussed, in order to predict equilibrium and growth shapes of related crystals. A short commentary about calcium carbonates chemistry and crystallography will be proposed and a brief summary about crystalline growth theories and computational crystallography will be provided. Techniques used for growing real crystals will be explained and obtained results will be proposed. Crystallization experiments, performed both in pure and calcium carbonate contaminated environment, will be compared, in order to show how the two substances, an organic one and an inorganic one, could interact.

INTRODUCTION

Fool: Canst tell how an oyster makes his shell?

King Lear: No.

Fool: Nor I neither.

[W. Shakespeare - King Lear]

Biom mineralization

Biom mineralization is an extremely complex process through which living beings interact with inorganic matter and modify its properties.

Without any doubt shells are the most known example of biom mineralization: through many phenomena mollusks are able to induce and control calcium carbonates crystallization processes and realize complex systems, completely different from similar materials from geological derivation. Shape is the most evident property: shells show very controlled and regular profiles, very well reproduced between members of the same species and very variable from species to species. Shapes like those very hardly can be defined random and it is the result of an evolution process that selected individuals able to produce structures with favorable hydrodynamical, mechanical or aesthetical properties that better satisfies biological requirements, like movement, defense, and mimicry and so on, with minimal material usage to avoid excessive weight and waste of energy and resources.

Another clear property is the high resistance: a bio-mineralized calcium carbonate can bear three thousand times the stress of the corresponding geological one. This objective is achieved thanks to textures, which allow distributing locally applied loads all over the system in a very effective way. The effect comes from the inner characteristics of the material: crystalline structure and morphology and the unique way in which crystal are connected.

Hardly ever is a bio-mineral composed of a single crystal: better properties are achieved thanks to a multitude of nanometric and controlled crystals. Single nano-crystals may have a very different morphology from the geological and inorganic one. Crystallization control involves also spatial orientation of the crystallites: these multitudes of crystals produce a typical X-ray diffraction (XRD) pattern, very similar to the one of the single crystals, proving that nanoparticles are all oriented in the same way.

Those nano-crystals are strictly interconnected and immersed in an organic network made of proteins, glycolides and/or lipids, produced by the

living beings. Joint system is able to exploit stiffness and hardness of the mineral, limiting brittleness and providing resistance and resilience thanks to a soft and elastic matrix, which can damp impacts and external loads.

Regular disposition of nanometric crystals provides also very interesting optical properties: opalescence of mother pearl, for example, is due to its acting like photonic crystals.

Another crucial phenomenon involved with biomineralization is polymorphism control: phase of the substance of which bio-mineral is composed of is quite usually thermodynamically unstable. Living beings select which phase to introduce in their structures, which may become complex due to spatial abrupt phase change in much closed regions. These phases become very stable and more corrosion resistant respect to their geological corresponding.

Back to shells example, those ones are able to withstand the marine environment for many years, resisting to corrosion and abrasion: one should think about difficulties in developing material for similar environments, like steel, concrete or plastics. Shells, with the exception of an organic matrix and cover, are composed of nothing but calcium carbonate. Most common phase in shells is aragonite, normally unstable, and vaterite, even less stable, is also present; in most cases, there are layers of calcite, on the external side, and vaterite, in a regular and perfectly controlled and with spatial sharp phase transitions

All these processes take place in aqueous environment, very far from being pure and at room temperature and pressure: results achieved in these bland conditions are incredibly better compared to what is artificially reachable in clean rooms and prohibitive thermodynamical conditions.

If one could reproduce, even only in a small portion, a crystalline control similar to the one performed by simpler organisms an enormous leap will be done in material design and production; artifacts with better properties and less weight, like shells, would be available. Possible applications could be found in all fields with no limits, from prosthetics to building, from transportation to electronics.

Biomineralization Examples

Nature shows a great number of biomineralization examples, with various functions and various ways in which this objective is achieved. Main

substances involved in biological controlled crystallization are calcium carbonates, and phosphates and silicates. The first ones are usually in their crystalline phases, while silicates are usually amorphous. All those substances have normally low solubility and for this reason it is very difficult to obtain regular crystals and play with super-saturation conditions; this is another clue point in favor of biological world, which can maintain a strict and dynamical control over very low concentrations ($[Ca^{2+}] \approx 10^{-7}M$).

The main function of bio-minerals is to provide very reliable mechanical properties; this is the role of sustain of bones and skeletons in all vertebrate: it is a composite system based on hydroxyapatite, alias calcium phosphate, extremely elastic and resistant, able to withstand continuous loads and strong shocks. Birds' eggs, made by a thin layer of calcium carbonate that in right conditions is able to withstand very high stresses, provide another example. Also sustains realized by weeds and corals in carbonates and silicates must be remembered.

Mechanical hardness and consumption resistance are the basis of all bio-minerals involved in biting, cutting and milling; teeth are made of fluorine-hydroxyapatite and are characterized by a complex inner microstructure despite exterior smooth and shiny aspect of their surface.

A typical role of bio-minerals in plants is ionic reserve and buffer, used to maintain a fixed chemical concentration; this objective is typically played by amorphous calcium carbonate.

Another property usually exploited by aquatic creatures is weight: bio-mineral density is a key point in floating devices and gravimetric sensors. Different densities due to the different packing of atoms or molecules unavoidably characterize different crystalline phases of a substance. Controlling crystallization of the various phases allows realizing very sensitive devices, like bio-inclinometers in fish ears; those ones are made of the three crystalline polymorphs of calcium carbonate, spatially separated. Also human equilibrium sense is based on calcium carbonates bio-minerals.

Remaining in devices and sensors field, optical and magnetic properties of bio-minerals are very important.

The lens of the first animals with sight organs, such as trilobites, consisted of one single crystal of calcite. This mineral is characterized by phenomena of birefringence, which are missing if the light passes through the crystal along particular directions. The crystallization of calcite in the eye of trilobite occurred to control the spatial orientation, in order to ensure this alignment to avoid the animal a double vision of the world.

Within particular bacteria, organelles containing a crystal of magnetite with a single magnetic domain were observed: the various organelles are aligned with each other as in a chain that allow the microorganism to be anchored to paramagnetic materials, besides allowing the perception of the magnetic fields and therefore the orientation in space, exactly like a compass.

Control Mechanisms

The organism effectively controls everything related to the formation of crystals. For example, to control the size, a spatial confinement around the core is created, a role that can be played by a vesicle. Supersaturation and flow of matter are other parameters to be monitored: the biological system manages the concentration of the chemical species involved in crystallization. There are several ways in an organism to adjust the concentration, some of these are similar to those used during the crystallization experiments, and others are peculiar of living beings: among them, the most important is certainly the mechanism of pumps and ion channels, fundamental for cell life.

The biological system must also provide preferential nucleation sites, to control the orientation and the global organization of the various crystallites, function that can be performed by an organic matrix that exposes an ordered set of functional groups. It is possible that there be a real epitaxy between the lattice of these groups and that of the inorganic phase that nucleation, which will do so with a very specific crystallographic orientation. This is also a first possible selection system of the polymorphic modification of inorganic or crystalline habit.

Last but not least, these two elements of control will be the main topic of this work: the living organism has the ability to induce the formation of a specific phase and with a different crystal habit from that found in the geological world, entering the interface with the solution substances that behave as impurities. In particular these may alter the shapes of equilibrium and growth of inorganic phases, by binding to surface sites of the latter, and then changing energies surface and attachment. More interaction can be achieved if, as in the case of the support matrix, to achieve epitaxial growth between the crystal lattice of the impurity released. The last step from the point of view of the affinity between impurity and the growing crystal is the coincidence between the thicknesses of the lattice planes, that is to say an epitaxy also in the third dimension: in this case released substance may even be incorporated within the inorganic material.

A living system is characterized by the simultaneous presence of very complex substances, such as enzymes and macromolecules, as well as from the occurrence of the contemporary all phenomena described so far, in a large and intricate interplay of event. From here the extreme difficulty of the study and understanding of biomineralization arises, which increases dramatically if you attempt to reproduce these phenomena in the laboratory.

General Idea of This Work

In the last few years in scientific literature there are many works on the phenomenon of biomineralization and changes in crystal habit or phase precipitated as a result of phase mother of substances somehow related to the biological world. It goes from simpler components such as fundamental monomers related to the life (monosaccharides, amino acids, DNA nucleobases ...), to the addition of complex proteins, enzymes and fragments of cross-linked macromolecules extracted directly from biological systems that realize biomineralization.

The idea behind the study carried out in of this dissertation is quite simple. It has been chosen the inorganic system most common in the world of biomineralization, that of calcium carbonates, and one of the simplest substances at the base of the molecules of life, an amino acid. It was chosen the α -alanine, the simplest chiral amino acid, among the twenty proteinogenic ordinary: a large part of living systems is based on the L amino acids, so this was preferred enantiomer.

Rather than focus observations on changes occurring on the inorganic material in the presence of alanine as an impurity the exact opposite was analyzed, i.e. changes habit crystalline of alanine in presence of CaCO_3 . Wishing to investigate reasons of crystallographic nature, as can be an epitaxy, at the base of the phenomenon of selectivity, we tried to observe the effects by another point of view. This pathway has been chosen by assuming that, given two substances, if the first was able to realize epitaxial growth on the second, the second may also behave in the same way on the first.

To do this it was first necessary to know in greater detail the characteristics of the crystals of alanine obtainable in pure environment, both from a theoretical point of view, and both from the experimental one: the shape of organic crystals, rarely observed in nature, is less well known than that of carbonates, abundant in minerals from around the world. To calculate theoretically the morphologies of the crystals of alanine was necessary to

develop a potential function capable of describing the energy properties of the molecular packing; experimentally it was necessary to prepare crystals of sufficient size and of good quality, to be able to observe the habit and the surface.

At this stage crystals of the same amino acid were prepared from a mother phase containing calcium carbonate as impurity, in order to observe a change. This phenomenon can be easily correlated to the opposite ones, which involved in biomineralization processes.

PART A. THEORETICAL PREDICTION ABOUT ALANINE CRYSTALS MORPHOLOGY

There is so much pleasure to be gained from useless knowledge.
[B. Russell]

In this first part, we try to predict, on the basis of the crystallographic structure and geometric considerations and energy, which are the shapes equilibrium and growth that characterize alanine crystals.

Over the following chapters the directions of strong bond and possible faces delimiting the equilibrium shape are first identified. By *ab initio* calculations is evaluated the interaction energy between a few molecules in the crystal, values that are used in building of a semiempirical potential. With this potential surface energy of the forms identified above is calculated and a profile for the equilibrium shape is finally drawn.

Afterwards, thanks to the calculation with the same potential energy of attachment, two models for the growth form are proposed.

L- α Alanine Crystalline Structure

Space Group

The α -alanine crystallizes according to the orthorhombic space group $P2_12_12_1$. This space group does not contain the inversion center, which would not be compatible with only one isomer of a chiral molecule like the one at issue. For this purpose, it may be useful to note that the racemic mixture of DL alanine crystallizes in a space group center-symmetric, although in the course of this thesis we will not deal with this subject.

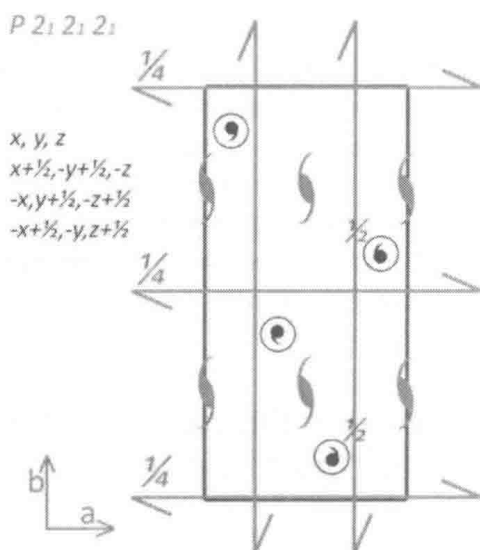


Figure A-1.1. Space group that owns the Crystal of L- α -alanine and its equivalent positions.

The space group of L-alanine, the number 19 International tables, is characterized by three elements of symmetry: these are three axis of rototranslation of order two, parallel to the three Crystallographic axes. In fact the Group generators are just two, in fact equivalent positions within the cell are only four, as shown in Figure A-1.1.

The three axes of symmetry do not intersect: the intersection between the two of them would generate an inversion Center. Furthermore, since improper axis, must be present in odd number within the group.

The point group of the structure is the $2_2 2_2$, which is obviously not center-symmetric: the dress of the Crystal will satisfy this symmetry.

The L-alanine molecule has an electric dipole moment: the presence in the cell four elementary equivalent for rotationally symmetrical molecules cancel all three components of the dipole, the elementary cell.

Crystallographic Parameters

The work that follows is based on structural determinations of c. Wilson et al. of 2005: the Atomic positions are obtained from neutron diffraction and this also allows obtaining reliable coordinate for the hydrogen atoms. The data were obtained with a spallation source and time-of-flight detector (TOF) and subsequently refined by Rietveld analysis.