Molecular Crystals and Liquid Crystals Incorporating Nonlinear Optics
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl17

Molecular Recognition in Pathological Crystallizations: Gout. Interactions between Albumin and Sodium Urate Mono-hydrate Crystals

D. Perl-treves [a] & L. Addadi [a]
[a] Structural Chemistry Dept., Weizmann Institute of Science, Rehovot, Israel
Version of record first published: 22 Sep 2006.

To cite this article: D. Perl-treves & L. Addadi (1990): Molecular Recognition in Pathological Crystallizations: Gout. Interactions between Albumin and Sodium Urate Mono-hydrate Crystals, Molecular Crystals and Liquid Crystals Incorporating Nonlinear Optics, 187:1, 1-16

To link to this article: http://dx.doi.org/10.1080/00268949008036022

Mol. Cryst. Liq. Cryst., 1990, vol. 187, pp. 1–16
Reprints available directly from the publisher
Photocopying permitted by license only

Printed in the United States of America

MOLECULAR RECOGNITION IN PATHOLOGICAL CRYSTALLIZATIONS: Gout. Interactions between Albumin and Sodium Urate Monohydrate Crystals

D. Perl-Treves and L. Addadi
Structural Chemistry Dept., Weizmann Institute of Science, Rehovot, Israel.

Abstract: Pathological crystallizations encompass a range of diseases caused by the formation of unwanted crystals in the body fluids. We investigate the relations between these crystals and proteins present in the biological environment, aiming at a basic understanding of their interactions at a molecular level. We focused on the crystallization of monosodium urate monohydrate (MSU), related to the disease of gout. Human serum albumin (HSA), which is found associated with the crystals in gouty patients, acts *in vitro* as a crystal nucleator of MSU. Morphological and immunolabeling studies showed a selective interaction of HSA with the {1$\bar{1}$0} faces of MSU crystals. These faces are formed of homocharged layers of sodium cations or urate anions. The nucleating effect is pH dependent, with the mid-point of the curve around physiological pH. Blocking reactions indicate that carboxylate groups are active in the nucleation of MSU. We propose a nucleation mechanism whereby structured anionic domains on the protein surface interact with cationic layers on the (1$\bar{1}$0) crystal surface. This type of interaction may be general for induction of crystallization under pathological conditions.

The importance of the role played by crystals in our body and in the life of most organisms is in general not fully appreciated. Organisms from all five kingdoms are known to build solid phases that fulfill a large variety of functions, ranging from skeletal support to food grinding and protective structures, from navigational and balance purposes to storage sites. In many cases, the organisms are able to control the mineralization process at the molecular level[1].

In contrast to biomineralization, pathological crystallization refers to the formation of unwanted crystals inside the organism. Common examples are kidney stones, formed mainly of calcium oxalate, uric acid and brushite crystals, or gall stones, composed mainly of calcified deposits of cholesterol. Certain types of joint diseases are also associated with the deposition of crystals, namely sodium urate crystals in gout, calcium pyrophosphate crystals in pseudogout, or apatite crystals in osteoarthritis[2]. Pathological crystallizations cannot however be classified according to chemical composition only. The crystalline deposits display a variety of polymorphic forms, different crystal habits and aggregation states, each probably reflecting a different etiology.

In all these diseases crystals grow in complex biological environments and interact with their molecular and macromolecular constituents. Often whole arrays of compounds are found associated with the pathological crystals, and it is practically impossible to distinguish compounds that may have triggered crystal formation from those that adhered to the crystals at a later stage. It is thus a very difficult task to determine the mechanism(s) of nucleation and growth of these crystals at the molecular level.

In all these diseases the body fluids are supersaturated with respect to the mineral formed at the site of deposition: This however is a necessary but not sufficient condition for the disease to occur[2] indicating that, as always in crystallization, the environment has an essential role in the triggering and/or modulation of crystal growth. Additives have long been known to exert an inhibitory effect on crystal growth, as they are adsorbed onto the crystals or nuclei, and thus interfere with their regular growth[3]. On the other hand, nucleation is rarely an homogeneous process; organized surfaces of monolayers[4], polymers[5] or proteins[6] have been shown to operate, under appropriate conditions, as nucleators of various crystalline materials at different levels of specificity.

In biomineralization, specific macromolecules are secreted by many organisms in order to initiate and control crystal formation[7]. In pathological crystallizations, on the other hand, epitaxial nucleation

on specific biological substrates is not expected, since the biological environment is not predetermined to interact with the crystals. Nucleation on these substrates is rather expected to follow rules of induction at some lower level of specificity, which may well present some general common characteristics. We feel that a basic understanding of the ways of interaction between forming crystals and the macromolecular components in the biological environment must first be achieved before addressing the question of the molecular mechanism(s) involved in pathological crystallizations.

Crucial to the treatment to follow is the concept that crystal surfaces, although determined by the arrangement of the molecules within the crystal lattice, display surface structures which are different from each other and from that of the crystal bulk. Interactions, at any level of specificity, must be therefore influenced by the structure of the surfaces of the crystal and of the interacting counterpart[8]. We shall hereforth describe the approach we have developed in the study of systems relevant to pathological crystallizations, where crystal-protein interactions are analyzed at the level of recognition between structured surfaces.

For an analysis of recognition patterns between macromolecules and crystals, ionic molecular crystals have many advantages over inorganic ones; they are stereochemically more articulated and the density of charge on their surfaces is lower. Specific interactions are thus easier to distinguish from non-specific electrostatic adsorption. These are some of the reasons why we chose to study sodium urate monohydrate (MSU) crystals, whose precipitation in joints causes the disease known as gout. The crystal structure of MSU is known[9], and possible factors influencing MSU crystallization have been investigated in a large number of clinical, as well as laboratory studies[10-14]. The factors predisposing certain hyperuricemic patients and not others to develop gout are, however, still unknown[2].

Sodium urate monohydrate crystals, formed in vitro and in the joint fluid (synovial fluid), appear as elongated plates of 1 to 50 μm in length[15]. The crystals grown in vitro display well defined large {010} plate faces, narrow {100}, {1$\bar{1}$0} and occasionally {110} side faces, and small {001} top faces (Fig.1)[16]. This

morphology indicates faster growth in the c direction relative to the directions perpendicular to c. This can be easily rationalized, as the packing is characterized by strong stacking interactions of the purine rings along c, reinforced by electrostatic interactions with the sodium ions arranged in the interstack space (Fig.2a). Significantly, potassium urate is isomorphous to MSU, while the corresponding salts of lithium, cesium or rubidium couldn't be obtained under analogous conditions. This is presumably due to the match between the spacing along c as determined by the stacking interactions and by the sodium, or potassium ion. A similar match cannot be achieved with the small lithium ions or with the large cesium ones.

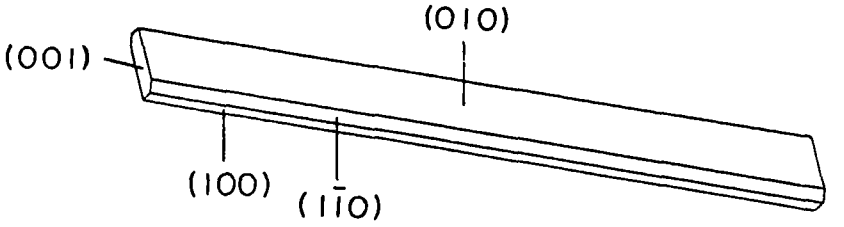

Fig.1: Morphology of typical crystals of monosodium urate monohydrate grown in vitro at room temperature, pH 7-8.

The small {001} faces (Fig.2e) have the strongest hydrophobic character, while {010} (Fig.2a) and {100} (Fig.2b) have an intermediate ionic character with purine rings alternating along the face with sodium ions and water molecules. The {1$\bar{1}$0} faces have a unique character; they are composed of layers of sodium cations and water molecules (Fig.2d) that run parallel to the face, alternating with layers composed exclusively of urate molecules (Fig.2c). Each molecule of urate emerges edge-on to the layer, thus endowing it with an anionic character.

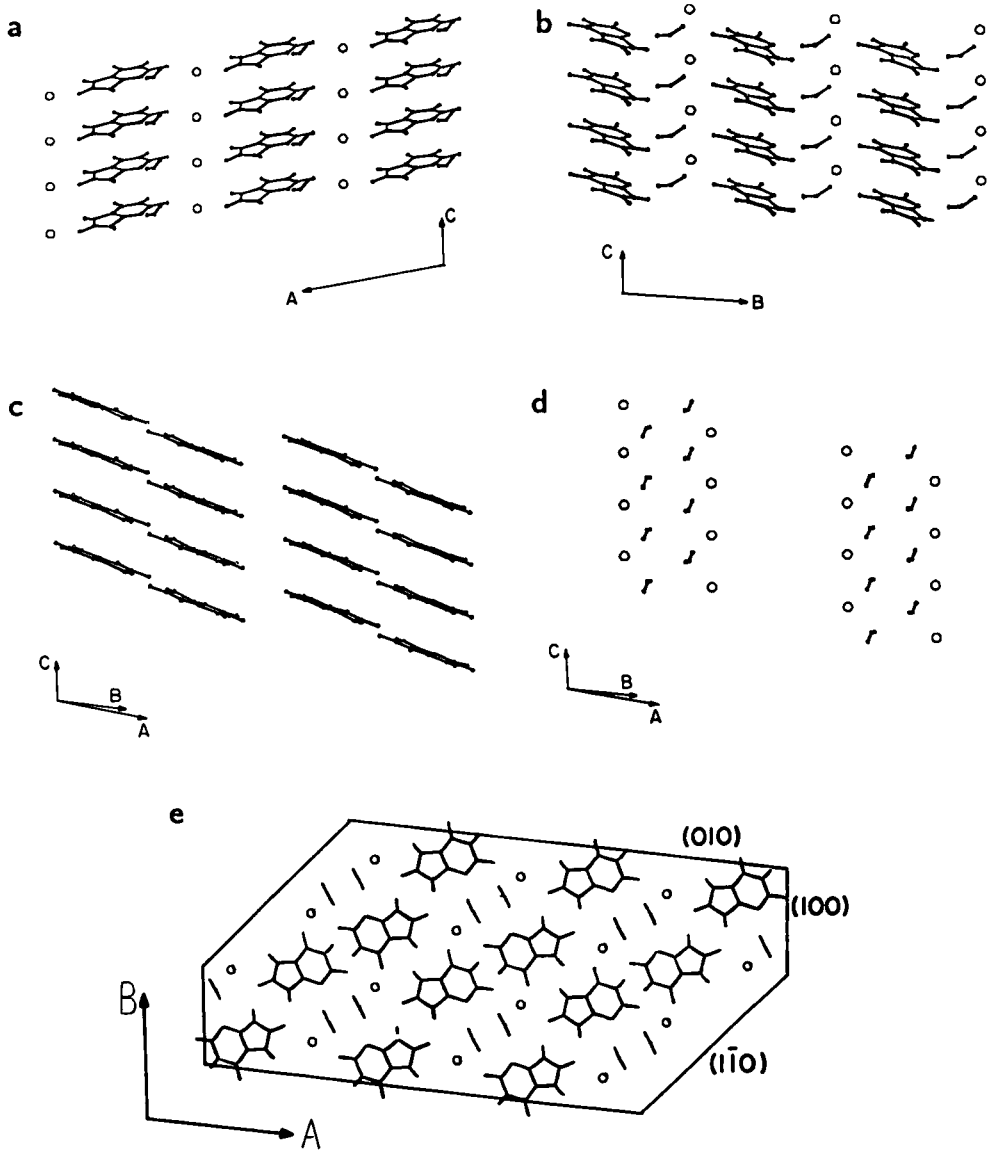

Fig.2: Packing arrangement of monosodium urate monohydrate: (a) on the (010) face; for clarity, only one molecular layer is shown. (b) On the (100) face; for clarity only one molecular layer is shown. (c) on the (1$\bar{1}$0) face; the layer of urate ions. (d) on the (1$\bar{1}$0) face; the layer of Na^+ ions and water molecules. (e) on the (001) face, delineated by the expressed side faces.

In vivo, MSU crystals are associated with a variety of synovial fluid components, but mainly with albumin and immunoglobulins[17]. In this study on crystal-protein interactions, we chose to first investigate in vitro the system MSU-albumin. Human serum albumin is the main protein component of serum and synovial fluid (conc. 6-20 mg/ml). It is a globular protein of MW=66 Kdaltons that is involved in a wide range of transport processes in blood. It has some polyelectrolyte character, due to the presence of a relatively large number of acidic and basic residues at its surface[18].

We found that albumin accelerates up to tenfold the appearence of crystals of MSU in vitro relative to the same solutions in the absence of albumin, and that the process is exclusively kinetic[19]. Heat-denatured albumin loses most of its nucleating power, thus highlighting the importance of the protein surface structure in the nucleation process. In addition, albumin has no effect on the nucleation of uric acid crystallized under the same conditions (pH=7-8, room temperature) but in the absence of sodium. It does, however, accelerate the crystallization of potassium urate, which is isomorphous to MSU. These results indicate a high degree of specificity of the nucleation effect in relation to both the protein and to the crystal surface structure. Studies were then undertaken to further define the specificity of the protein-crystal contacts.

CRYSTAL SPECIFICITY

Three independent techniques were used to determine which, if any, of the crystal faces, preferentially interact with the protein; morphology, immunofluorescence and immunogold labeling[19].

Morphology: The crystal habit appears to be unchanged when crystallization is performed in the presence of albumin in solution. The relative area of the side faces does, however, increase, indicating that adsorption of protein takes place preferentially on the {1$\bar{1}$0} and/or {100} faces.

Immunofluorescence: Crystals grown in the presence of protein were incubated with antibody raised against human serum albumin, and subsequently with fluorescently labeled Protein A. The latter protein selectively binds to antibodies. Fluorescence was detected specifically from the side faces of the crystals grown with albumin in solution, indicating the selective presence of albumin on those faces (Fig.3). No fluorescence was observed from the control crystals grown without albumin. Denatured albumin appears by this test to induce a much weaker response, and fluorescence seems to be equally distributed on all crystal faces.

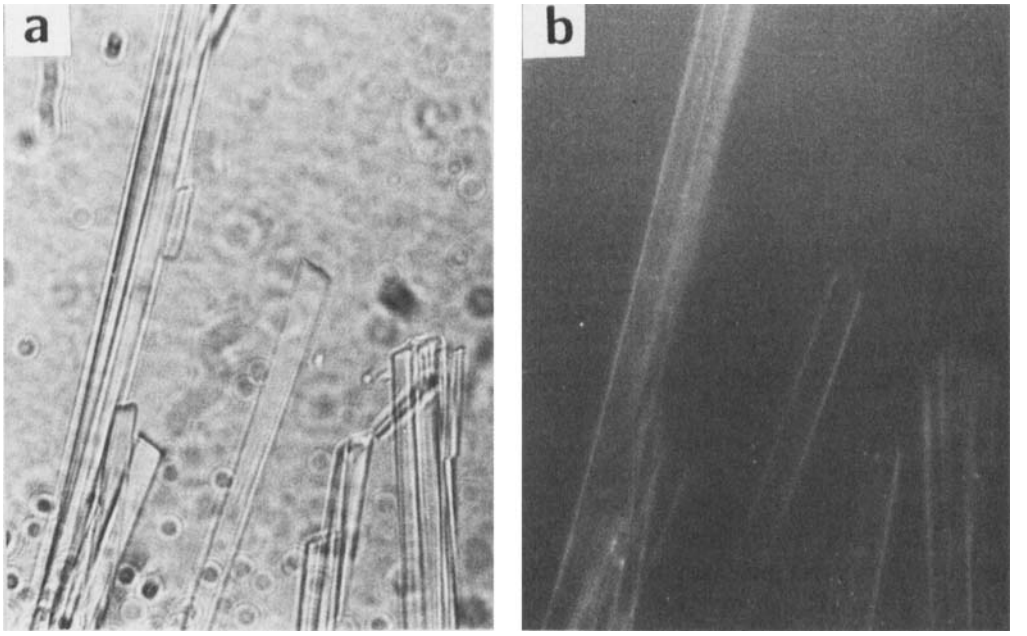

Fig.3: Immunofluorescence labeled crystals of monosodium urate monohydrate (a) viewed in white light, (b) viewed in the fluorescence mode: Enlargement x800.

Immunogold labeling: Crystals incubated with antibodies as described above were subsequently incubated with colloidal gold labeled Protein A. The crystal faces were identified in the scanning electron microscope, and the number of gold particles adhering to each of them was counted. On the basis of observations performed on a large number of crystals, one face type, {1$\bar{1}$0}, interacts

preferentially with albumin. As noted above, this face has the unique characteristic of being formed of homocharged layers alternating parallel to the face. The protein domain interacting with such a face must also be charged. We describe below the information obtained on the nature of the groups composing these domains.

PROTEIN SPECIFICITY

The nucleating effect of albumin towards MSU crystals was found to depend strongly on pH[19]. The critical region is between pH 7 and 8. Albumin is barely nucleating at pH 7 and displays almost maximal acceleration of nucleation above pH=8 (Fig.4). This effect hints at the participation of groups whose state is changed around pH 7.4. Such modifications in proteins may be due either to direct titration of the active groups, resulting in a net change of charge, or to conformational changes.

A series of studies were undertaken to identify the groups involved. Blocking of lysine residues with dansyl chloride had a weak erratic effect on the nucleating power of the resulting blocked proteins, although as much as 30 groups out of a total of 52 were blocked (Table 1). Blocking of carboxylic groups performed at pH=6 with glycine ethyl ester, under non-denaturing conditions, resulted in 48 groups being blocked. The blocked protein had its nucleating potential reduced by 50%. The reagent acts on neutral carboxyls and has an optimum operating pH at 6. Reaction at higher pH resulted in fewer groups being blocked and in a concomitant decrease in the effect of the blocking on nucleation[20].

Blocking of carboxylate groups by Woodward's reagent K reduced the nucleating effect of the reacted albumin by up to 95% when performed at pH=7.6. The influence of blocking was also dependent on the number of groups blocked (Table 1). Interestingly, when the reaction was performed at pH=7, the nucleating effect was reduced by only 8 %, even though the nucleation experiment was performed at pH=8. Since the reaction was not accompanied by denaturation in either case, the difference must be attributed again to the nature of the titratable groups. There are $\sim$93 amino acids with carboxylate side chains in albumin, most of which are thought to be exposed to water. With 16-28 groups reacted, blocking must take place statistically on the

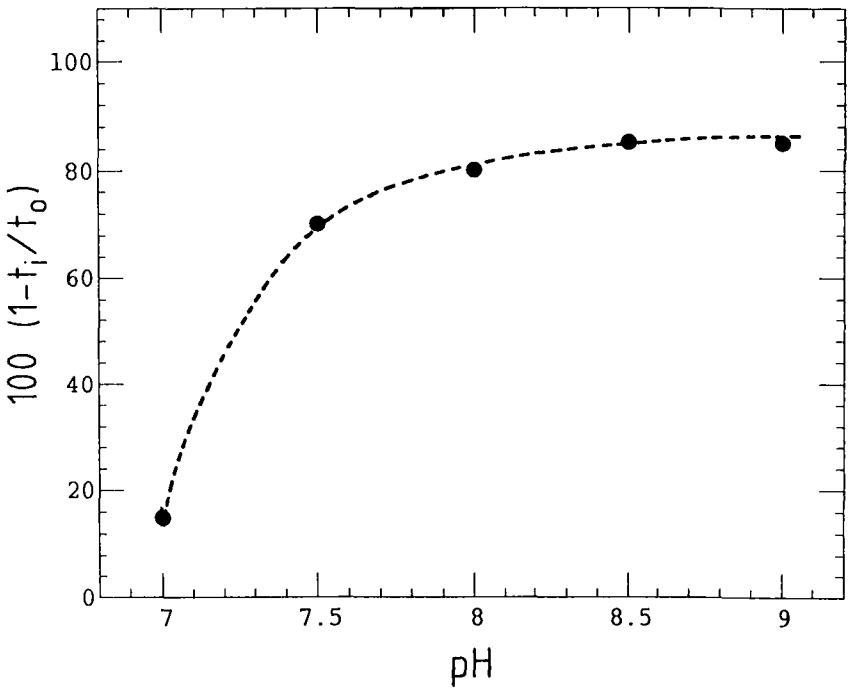

Fig.4: pH profile of the effect of albumin on sodium urate nucleation. The nucleating power is expressed as 100 (1 - t_i/t_o) where t_i and t_o are the induction times for nucleation in the presence and in the absence of albumin respectively.

exposed groups. We can infer that deactivation of the protein with respect to nucleation occurs when one or more carboxylate groups of the nucleating domain is blocked. These groups are susceptible to blocking at pH=7.6, but not at pH=7.0. The results are thus in agreement with the pH dependence of the nucleating effect. It has been suggested[21] that Woodward's reagent K reacts with carboxyls only when they are in the charged form COO^-. If this is true, the pKa of the groups active in nucleation would be set around 7.4, as compared to the pKa of an isolated carboxyl which is around 4. In biological environments[22] and on surfaces[23], however, pKa values of carboxylic groups ranging from 3 to 11 have been reported. In view of this, a pKa of 7.4 is not

Table 1

Nucleating effect of HSA at pH= 8.0 after blocking of HSA carboxyl groups with glycine ethyl ester (GEE), dansyl hydrazine (DNS-H) or Woodward's reagent K (WRK), and amino groups with dansyl chloride (DNS-Cl).

Reagent	pH of blocking reaction	nature of the groups	number of groups blocked	residual nucleating power [a]
GEE	6.0	COOH	48	0.52
GEE	7.0	COOH	20	0.89
GEE	8.0	COOH	16	1.00
DNS-H	7.0	COOH	?	0.72
WRK	7.6	COO^- [b]	16	0.85
WRK	7.6	COO^- [b]	20	0.50
WRK	7.6	COO^- [b]	28	0.05
WRK	7.0	COO^- [b]	20	0.92
DNS-Cl	8.0	NH_2	18	0.97
DNS-Cl	8.0	NH_2	30	1.00

a: $(1-t_b/t_o)/(1-t_n/t_o)$. t_o: time of appearance of the crystals in the absence of HSA, t_b: in presence of blocked HSA, t_n: in presence of native HSA.
b: suggested.

---

particularly surprising. It would imply closely spaced domains of negative charges influencing each other. Alternatively, the observed pH dependence may be explained by conformational transitions. Indeed, between pH 6 and 8 human serum albumin undergoes the so called 'neutral transition'[24], and an active participation of carboxylate groups in this transition has been proposed[25]. The nucleating carboxylate groups in this scenario may thus be exposed at pH=8 and 'hidden' at pH=7.

It has been suggested that Woodward's reagent K may react at higher pH with amine or imidazole groups, in addition to carboxylates[26]. This possibility gives rise to a different explanation of the pH dependence of the nucleating effect, involving histidines and/or lysines in their neutral basic form. The results obtained by blocking with dansyl chloride and glycine ethyl ester, do however show involvement of carboxyl, and not amino groups. In addition, as established above, the interacting crystal faces are charged, implying that the counterpart should be charged as well. Involvement of basic residues would thus be expected to result in an opposite pH dependence of the nucleating effect.

IMAGE OF THE NUCLEATION SITE

It has been tacitly assumed in the discussion to this point that specific structural protein/crystal interactions occurring during nucleation follow the same recognition rules as those occurring during growth. This implies that the nuclei have the same structure as the mature crystals, a generally accepted assumption strengthened by some indirect experimental evidence[27-28].

If this assumption is correct, then combined with the data on crystal specificity and protein blocking reactions, certain conclusions can be drawn about the nucleation site, and a tentative nucleation mechanism can be consequently proposed. The nucleation site is composed of a structured domain of carboxylate groups on the protein surface, whose relative geometry is determined by the secondary and possibly tertiary structure of albumin. This structured anionic nucleating domain interacts with a layer of sodium ions and water molecules from solution, akin to the cationic layer of MSU crystals on the $(1\bar{1}0)$ face. Whether this first layer is assembled on the protein or is part of a metastable molecular aggregate preexisting in the supersaturated solution is open to speculation. Given the very high level of supersaturation of solutions of sodium urate that can be maintained in a metastable state without nucleation[29], we personally prefer the latter explanation. The function of the protein would then be to stabilize the nucleus, allowing it to eventually grow into a crystal.

The three dimensional structure of albumin has only very recently been determined at a resolution of 6 Å[30]. It is composed of a series of α-helical domains assembled in tight packing to yield an extended fairly planar ellipsoidal surface of 140 by 40Å. Unfortunately the side chains of the amino acids cannot be identified at this resolution. The known sequence of albumin however includes series of aspartate and glutamate residues located such that they would be very close to each other if arranged in an α-helical structure.

The proposed nucleation mechanism is reasonable in terms of pathological crystallizations, insofar as it involves the interaction of anionic domains on the protein with cationic layers on the crystal. This type of interaction does not require epitaxial matching of atomic positions and can be quite general for nucleation of ionic crystals on charged substrates. The same essential features were deduced for nucleating domains on mollusk shell acidic proteins[6,7]. In this case the mineral is calcite and the crystals nucleate within a preformed solid matrix. Analogous to sodium urate, the first layer of calcium cations of the crystals interact with structured carboxylate domains of aspartic and glutamic acid residues. Nucleation of calcite ensues, with a specific orientation corresponding in the crystal lattice to layers of calcium cations and carbonate anions perpendicular to the c axis. Similarly oriented crystals of sodium chloride[4] and vaterite[31] were observed to form under monolayers of stearate. Such striking similarities in systems that are so different testifies to the generality of the proposed nucleation mechanism.

We note in this context that the acidic nucleating proteins extracted from mollusk shell matrix, nucleate calcite only when adsorbed on a substrate. These are small proteins that assume an extended β-sheet conformation. The substrate, be it their natural matrix framework or an artificial one, provides the protein with the rigidity required for nucleation to occur. The same requirement is apparently not necessary for a large globular protein as it is sufficiently rigidized by intramolecular interactions. In fact albumin is known on the contrary, to undergo a certain degree of denaturation upon adsorption to substrates[32].

We found that albumin adsorbed on glass preserves its ability to nucleate MSU crystals. In contrast, albumin adsorbed on plasma-treated polystyrene does not induce crystallization of MSU any faster than polystyrene alone. The different behaviour of the protein can clearly be attributed to the chemical nature of the surface. Albumin must be denatured to a greater extent when adsorbed on the hydrophobic polystyrene surface, than on the more hydrophilic glass. We are presently investigating the mechanisms of adsorption-denaturation-nucleation relations in greater detail[33]. These studies are also relevant to the pathological condition, where crystallization takes place in contact with the charged surface of cartilage, comprising collagen and proteoglycans[2].

CONCLUSIONS

We have shown here that proteins present in the body fluids can recognize different crystal surfaces on the basis of their structure and chemical nature. Recognition can result in selective adhesion of the macromolecule to the crystal surface and in stabilization of crystal nuclei towards crystallization. In the case of gout, the mechanism suggested here for nucleation *in vitro* may be relevant to the *in vivo* situation as well. Albumin is always present in the synovial fluid, but it is not always in a nucleating state. We note that physiological fluids have pH values close to 7.4. This pH is at the mid-point of the observed pH dependence curve for nucleation of sodium urate by albumin. The combination of supersaturation with localized pH fluctuations may then transiently induce conditions favourable to nucleation. Furthermore, from studies performed directly on synovial fluids extracted from gouty and non-gouty patients, it appears that the behaviour of albumin with respect to MSU nucleation is the same in the physiological environment as in buffer solutions[20].

We do not intend to suggest that this is the only pathway to crystallization in gout. It may provide only one, out of many possible scenarios for the onset of crystal formation in the disease. On the contrary, we note that the same mechanism suggested for albumin may be operative for other proteins as well. Interestingly, we found in this context that the large family of IgG immunoglobulins also induce *in vitro* faster crystallization of MSU,

although a concentration of IgG higher than for albumin is required in solution in order to reach an analogous effect[20]. Immunoglobulins are the hypervariable family of proteins comprising the antibodies in the body fluids. They have the task of recognizing foreign particles invading the body. Pathological crystals are also included in this definition of foreign particles, and as such might be conceivably recognized by specific antibodies. We are presently investigating this interesting possibility[34].

Acknowledgements: We thank S. Weiner for critical reading of the manuscript, L. Leiserowitz for useful discussions. This work was supported by the Fund for Basic Research, administered by the Israel Academy of Science, and by the Minerva Foundation.

REFERENCES

1. H.A. Lowenstam and S. Weiner. In: On Biomineralization, Oxford University Press, New York London (1989).
2. P.A. Dieppe and P.D. Calvert. In: Crystals and Joint Diseases, Chapman and Hall, London (1983).
3. H.E. Buckley. In Crystal Growth,. Chapman and Hall, London (1951).
4. (a) E.M. Landau, R. Popovitz-Biro, M. Levanon, L. Leiserowitz, M. Lahav and J. Sagiv. Mol. Cryst. Liq. Cryst. 134: 323-325 (1986). (b) E.M. Landau, S. Grayer-Wolf, J. Sagiv, M. Deutsch, K. Kjaer, J. Als Nielsen, L. Leiserowitz and M. Lahav, Pure and Appl. Chem. 61: 673-684 (1989).
5. L. Addadi, J. Moradian, E. Shai, N.G. Maroudas and S. Weiner. Proc. Natn. Acad. Sci USA 84: 2732-36 (1987).
6. (a) L. Addadi and S. Weiner. Proc. Natn. Acad. Sci. USA 82: 4110-1414 (1985). (b) L. Addadi and S. Weiner. Mol. Cryst. Liq. Cryst 134: 305-322 (1986).
7. L. Addadi and S. Weiner., in: Biomineralization, Chemical and Biochemical Perspectives, Ed. S. Mann, M. Webb, R.J.P. Williams, V.C.H. Publ. Weinheim Chapter 5, (1989).
8. L. Addadi, Z. Berkovitch-Yellin, I. Weissbuch, J. van Mil, L.W.J. Shimon, M. Lahav and L.Leiserowitz. Angew. Chem. 24: 466-485 (1985).
9. N.S. Mandel and G.S. Mandel. J. Am. Chem. Soc. 98: 2319-2323 (1976).

10. B.T. Emmerson. Aust. NZ. J. Med. 18: 319-326 (1988).
11. H.M. Burt and Y.C. Dutt. Ann. Rheum Dis 45: 856-864 (1986).
12. H.K. Tak, S.M. Cooper and W.R. Wilcox. Arthr. Rheum. 23: 574-580 (1980).
13. W.R. Wilcox and A.A. Khalaf. Ann. Rheum. Dis. 34: 332-339 (1975).
14. P.D. Calvert, R.W. Fiddis and N. Vlachos. Colloids Surf. 14: 97-107 (1985).
15. K.P.H. Pritzker, C. Zahn, S.C. Nyburg, S.C., Luk and J.B. Houpt. J. Rheumatol. 5: 7-18 (1978).
16. C. Rinaudo and R. Boistelle. J. Cryst. Growth 57: 432-442 (1982).
17. P. Hasselbacher. In: Advances in Inflammation Research, Vol. 4, Ed. G. Weissmann, Raven Press , N.Y. (1982).
18. U. Kragh- Hansen. Pharmac. Rev. 33: 17-53 (1981).
19. D. Perl-Treves and L. Addadi. Proc. R. Soc. Lond. B 235: 145-159 (1988).
20. D. Perl-Treves (Ph.D. Thesis), Feinberg Graduate School, Weizmann Institute of Science, 1990.
21. B.M. Dunn and C.B. Anfinsen. J. Biol. Chem. 249: 3317-3727 (1974).
22. A. Fersht. In: Enzyme Structure and Mechanisms. pp. 143-154, W.H. Freeman Publ., Reading and San Francisco (1977).
23. S.R. Holmes-Farley, R.H. Reamey, T.J. McCarthy, J. Deutch and G.M. Whitesides. Langmuir 1: 725-740 (1985).
24. W.J. Leonard, K.K. Vijai and J.F. Foster. J. Biol. Chem. 238: 1984-1988 (1965).
25. B.J.M. Harmsen, S.H. De Bruin, J.F. Rodrigues de Miranda, L.H.M. Janssen and G.A.J. Van Os. Biochemistry 10: 3217-3221 (1971).
26. K. Llamas, M. Owens, R.L. Blakeley and B Zerner. J. Am. Chem. Soc. 108: 5543-5548 (1986).
27. I. Weissbuch, D. Zbaida, L. Addadi, L. Leiserowitz and M. Lahav. J. Am. Chem. Soc. 109: 1869-1871 (1987).

28. E. Staab, L. Addadi, L. Leizerowitz and M. Lahav. Angew. Chem. Adv. Mat., in press.
29. H. Furedi-Milhofer, V. Babic-Ivancic, O. Milat, W.E. Brown and T.M. Gregory. J. Cryst. Growth, 83, 572-580 (1987).
30. D.C. Carter, X.-M. He, S.H. Munson, P.D. Twigg, K.M. Gernert, M.B. Broom and T.Y. Miller. Science 244: 1195-1198 (1989).
31. S. Mann, B.R. Heywood, S. Rajam and J.D. Birchall. Proc. R. Soc. Lond. A 423: 457-471 (1989).
32. M.E. Soderquist and A.G. Walton. J. Colloid Int. Science 75: 386-397 (1980).
33. D. Hanein and L. Addadi. (Work in progress).
34. M. Kam, D. Perl-Treves and L. Addadi. (Work in progress).