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Crystallization of truncated human apolipoprotein A-I in a novel conformation

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The crystallization of recombinant human apolipoprotein A-I (apo A-I), the major protein component of high-density lipoprotein, in a new crystal form is described. The fragment crystallized, residues 44–243 of native apo A-I [apo Δ(1–43)A-I], is very similar to intact native apo A-I in its ability to bind lipid, to be incorporated into high-density lipoproteins and to activate lecithin–cholesterol acyl transferase. Apo Δ(1–43)A-I crystallizes, in the presence of β-D-octylglucopyranoside, in space group $I222$ or $I2_12_12_1$, with unit-cell parameters a = 37.11, b = 123.62, c = 164.65 Å and a diffraction limit of 3.2 Å. These form II crystals grow under conditions of significantly lower ionic strength than the original form I crystals (space group $P2_12_12_1$, a = 97.47, b = 113.87, c = 196.19 Å, diffraction limit 3.0 Å). Packing arguments show that the unusual open conformation of apo Δ(1–43)A-I found in the form I crystals cannot be packed into the smaller oddly proportioned form II unit cell. Monomeric apo Δ(1–43)A-I, as either a four-helix bundle (∼75 × 30 × 30 Å) or an extended helical rod (∼150 × 20 × 20 Å), can be packed into the form II unit cell. It is concluded, therefore, that apo Δ(1–43)A-I may have crystallized in one of these distinct conformations in the form II crystals.

1. Introduction

Apolipoprotein A-I (apo A-I) is the major protein component of high-density lipoprotein (HDL) particles found in the blood of humans and other vertebrates. High blood levels of HDL strongly correlate with a reduced risk of atherosclerosis and hence a reduced risk of coronary artery disease (Catapano *et al.*, 1993; Castelli *et al.*, 1986; Gordon *et al.*, 1977; Miller *et al.*, 1977). Of all of the constituents of HDL (triglycerides, cholesterol, apo A-I, apo A-II *etc.*), only apo A-I correlates with the protective effect of HDL (Schultz *et al.*, 1993; Warden *et al.*, 1993).

A major metabolic function of HDL is the reverse transport of cholesterol from peripheral tissues to the liver for catabolism (Miller, 1989). Apo A-I plays several key roles in this process (Rothblat *et al.*, 1992): it binds phospholipids to form nascent discoidal HDL; it promotes the reversible efflux of cholesterol from peripheral cell plasma membranes into HDL; it activates lecithin–cholesterol acyl transferase (LCAT), the enzyme which traps cholesterol within HDL by converting cholesterol to cholesterol esters, thereby transforming immature discoidal HDL into mature spherical HDL; it is responsible for the recognition of HDL by the HDL receptor, SR-BI, found in the liver and the adrenal gland (Acton *et al.*, 1993; Plump *et al.*, 1996; Kozarsky *et al.*, 1997;

Rigotti *et al.*, 1997). This latter process is the final step in the reverse transport of cholesterol. How apo A-I facilitates these processes is not understood at present.

The answers to the following fundamental questions regarding apo A-I and HDL structure and function would be provided, in part, by the appropriate crystal structures. (i) What is the tertiary structure of soluble lipid-free apo A-I? (ii) What is the structure of apo A-I bound to discoidal nascent HDL and to spherical mature HDL? (iii) What features of apo A-I facilitate the interconversion of these structures? (iv) How does apo A-I promote cholesterol efflux, activate LCAT and bind to SR-BI?

The structures of (apo)lipoproteins, especially apo A-I, have been intensively studied for decades (Scanu, 1972; Morrisett *et al.*, 1977; Brouillette & Anantharamaiah, 1995). Although much progress has been made, structural models of apo A-I have not changed significantly in the past 20 years, until our recent publication of the first crystal structure of apo A-I (Borhani *et al.*, 1997). The protein crystallized is an N-terminal truncation mutant of human apo A-I, 'apo Δ(1–43)A-I' (residues 44–243, corresponding to exon 4 of the apo A-I gene), which is very similar to native apo A-I (residues 1–243) in its ability to be incorporated into HDL and to activate LCAT (Rogers *et al.*, 1997). Apo Δ(1–43)A-I and native apo A-I exhibit similar lipid-binding properties, based on several lines of evidence: both associate exclusively with plasma HDL, show identical exclusion pressures in egg phosphotidylcholine (PC) monolayers, have similar free energies of binding to palmitoyl oleolyl PC vesicles and have similar α-helical contents when bound to lipid. Apo Δ(1–43)A-I retains structural characteristics of the lipid-bound protein even in the absence of lipid, whereas the structures of intact apo A-I in the presence and absence of lipid are distinct. For these reasons, we suggested that lipid-free apo Δ(1–43)A-I appears to be able to adopt and maintain, under certain conditions, the lipid-bound structure of apo A-I even in the absence of lipids (Rogers *et al.*, 1997).

It is widely believed that exchangeable lipoproteins such as apo A-I adopt a variety of conformations to enable them to carry out the varied functions described above. For example, the conformation of the lipid-free protein (Roberts *et al.*, 1997) appears to be distinct from the conformation of the lipid-bound protein (Rogers *et al.*, 1997; Borhani *et al.*, 1997). Indeed, Rogers *et al.* (Rogers, Roberts, Lebowitz, Datta *et al.*, 1998; Rogers, Roberts, Lebowitz, Engler *et al.*, 1998) have shown by ultracentrifugation that both apo A-I and apo Δ(1–43)A-I exist in a variety of interconverting conformational states: compact monomeric α-helical bundle $\rightleftharpoons$ extended monomeric α-helical rod $\rightleftharpoons$ open lipid-bound conformation. The molecular basis by which apo A-I is able to exist in several stable distinct conformations is not known. To build upon our first structural result, therefore, we have focused on the crystallization of apo A-I under different conditions, in part to observe these distinct conformations of apo A-I.

We report here the crystallization of apo Δ(1–43)A-I under new conditions (form II) which differ from our original conditions (form I) by the inclusion of a mild detergent and a significant lowering of the ionic strength. We present crystal packing arguments which suggest that the conformation of apo Δ(1–43)A-I in the form II crystals is indeed distinct from the unusual open 'horseshoe' conformation found in the high-salt form I crystals (Borhani *et al.*, 1997).

2. Materials and methods

2.1. General methods

Recombinant human apolipoprotein A-I (residues 44–243 plus a vector-derived N-terminal methionine residue, 'apo Δ(1–43)A-I', molecular weight 23264 Da) was overexpressed in *Escherichia coli* and purified by reverse-phase HPLC, as described previously (Rogers *et al.*, 1997). Greater than 97% of the protein was in the methionine-reduced form and remained in this form for up to 1 year when stored at 277 K in the presence of antioxidants. Purity and molecular weight were also assessed by discontinuous SDS–PAGE (Laemmli, 1970). Protein concentrations were determined by UV absorbance (extinction coefficient of 23078 M^{-1} cm^{-1}, $A^{1\%}$ of 9.92) at 280 nm in 6 *M* guanidine–HCl. This extinction coefficient was determined by quantitative amino-acid analysis.

2.2. Crystallization

Purified apo Δ(1–43)A-I (2.2 mg ml^{-1} in 10 m*M* HEPES, 50 m*M* NaCl, 1 m*M* EDTA, 3 m*M* NaN_3, 10 μ*M* α-tocopherol, 50 μ*M* ascorbic acid pH 7.5) was concentrated by ultrafiltration (Amicon YM-10 membrane) to 19.7 mg ml^{-1}. A preliminary screen for crystallization conditions was carried out with the Hampton Research Crystal Screen Kit under vapor-diffusion conditions at room temperature and 277 K (Carter & Carter, 1979; Jancarik & Kim, 1991). Two of 50 conditions (Nos. 22 and 38) provided crystals at 277 K only. Optimization of these crystallization conditions yielded two crystal forms. The form I crystals described previously grew from 1.0–1.4 *M* sodium citrate pH 6.5–7.5 (Borhani *et al.*, 1997).

2.2.1. Form II. 2 μl of apo Δ(1–43)A-I containing 0.25%(*w*/*v*) β-D-octylglucopyranoside were mixed with an equal volume of precipitant solution (16–24% polyethylene glycol 4000, 0.5 *M* NaOAc, buffered with 100 m*M* Na HEPES pH 7.5–8.0 or 100 m*M* Tris–HCl pH 8.5–8.75). The mixture was allowed to equilibrate at 277 K as a hanging drop on siliconized (Aqua-Sil, Pierce) glass cover slips over 1 ml of the precipitant solution (in 24-well Linbro plastic tissue-culture plates). The protein usually precipitated immediately; rod-like crystals grew from the precipitate in one week. These crystals, which have sharp edges but are usually hollowed-out from one end, grew to a maximum size of 800 × 100 × 100 μm over many weeks. Form II crystals appear to be stable in an artificial mother liquor consisting of 20% PEG 4000, 0.5 *M* NaOAc, 100 m*M* Na HEPES pH 8.0.

2.2.2. Flash-cooling. The form II crystals were equilibrated in their artificial mother liquor and then passed through mother liquor supplemented with 10 and 20%(*w*/*v*) glucose or sucrose (10 min in 10%, 5 min in 20%). Crystals were

mounted in rayon loops (Hampton Research) and flash-cooled by plunging into liquid nitrogen. Cracks developed about 5 min after the crystals were placed in the 20% glucose (sucrose) mother liquor. The rod-like crystals cracked perpendicular to their length. Sometimes, one large crystal broke into several shorter pieces, each of which still diffracted X-rays after flash-cooling.

2.3. X-ray diffraction measurements

2.3.1. Laboratory. Crystals were harvested either directly from the growth droplet or from the artificial mother liquor, and mounted in thin-walled glass capillaries (0.7 or 1.0 mm diameter). The X-ray diffraction characteristics of the crystals were determined at 282 K (forced-air apparatus) on a Siemens SRA rotating-anode generator (50 kV, 108 mA, graphite-monochromated Cu $K\alpha$ radiation) equipped with a MAR Research image-plate detector.

2.3.2. Synchrotron. X-ray diffraction data were collected at the National Synchrotron Light Source (beamline X25, λ = 1.100 Å, Brandeis B4 CCD detector). All data were collected by the rotation method (0.5–1.0°, typically 60 s exposure) at a temperature of 100 K. Some exploratory data were also collected at the Stanford Synchrotron Radiation Laboratory (beamline 7-1, λ = 1.080 Å, MAR Research image-plate detector) at 277 K. All data processing was performed with the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). The crystal orientations were determined with *REFIX* (Kabsch, 1993) and refined with *IDXREF*. The data were integrated with *MOSFLM* (Leslie, 1992), scaled and merged with *SCALA* (Evans, 1997) and placed on an absolute scale with *TRUNCATE* (French & Wilson, 1978).

2.3.3. Rotation function. The self-rotation function of the apo Δ(1–43)A-I form II crystals was calculated with *AMoRe* (Navaza, 1994). Data between 35 and 4 Å resolution were used in the calculation (NCODE = 1; Patterson integration radii, 20, 30 or 40 Å; Bessel orders less than 6 were omitted). The cross-rotation function was calculated similarly (at 6 or 4 Å resolution), using the structure of the form I monomer (Borhani *et al.*, 1997) as the search model.

3. Results and discussion

3.1. Crystallization of human apolipoprotein A-I in a new crystal form

The N-terminal truncation mutant of human apo A-I, apo Δ(1–43)A-I, was crystallized under two conditions. As described previously (Borhani *et al.*, 1997), form I crystals grew at 277 K as large bipyramids from sodium citrate, potassium citrate or sodium potassium tartrate pH 6.5–7.5. A new crystal form, form II, grew in a few weeks at 277 K as hollowed-out rods (800 × 100 × 100 µm) from PEG 4000 containing 0.5 *M* NaOAc and 0.25% β-D-octylglucopyranoside pH 7.5–8.75. The form I crystals did not grow in the presence of β-D-octylglucopyranoside, whereas the form II crystals grew only in its presence. Both crystal forms grew in the presence of precipitated protein.

Table 1
Diffraction data from an apo Δ(1–43)A-I form II crystal.

Resolution (Å)	Unique reflections	Coverage (%)	Multiplicity	$\langle I/\sigma_I \rangle$	R_{sym} (%)
50.0–9.05	282	85	2.0	15.8	7.9
9.05–6.40	498	90	2.3	16.3	3.5
6.40–5.23	630	90	2.3	13.9	3.7
5.23–4.53	665	84	2.1	12.8	3.9
4.53–4.05	676	77	2.1	10.0	4.8
4.05–3.70	736	76	2.0	7.1	9.8
3.70–3.42	808	77	2.0	4.8	15.0
3.42–3.20	887	78	2.0	2.3	40.5
50.0–3.20	5182	81	2.1	9.2	6.1

3.2. Data collection and crystal characterization

Like the apo Δ(1–43)A-I form I crystals (Borhani *et al.*, 1997), the new form II crystals were extremely sensitive to thermal damage and X-ray-induced radiation damage. With cooling to 277–282 K, the form II crystals diffracted X-rays feebly and briefly to a maximum resolution of 5 Å in the laboratory or 4 Å at the synchrotron. No useful data could be obtained prior to the definition of suitable flash-cooling conditions. Autoindexing of a single 1° rotation photograph suggested that this crystal form was body-centred orthorhombic (space group $I222$ or $I2_12_12_1$), with unit-cell dimensions of approximately 38 × 130 × 173 Å.

We examined whether cryogenic data collection would improve the poor diffraction characteristics of the form II crystals. Several cryoprotectants were tested. Glycerol, ethylene glycol and PEG 400 all failed to protect these crystals from disordering upon cooling to cryogenic temperatures. Glucose and sucrose did prove effective, although a completely satisfactory cryogenic mother liquor has not yet been found. The crystals tolerated a few minutes exposure to a 20% glucose (or sucrose) mother liquor, but then began to crack. Diffraction data were collected from the flash-cooled apo Δ(1–43)A-I form II crystals using synchrotron radiation. Under these conditions, the crystals diffracted X-rays to 3.2 Å resolution. The diffraction data are summarized in Table 1.

Unlike the form I crystals, cryogenic temperatures dramatically improved the stability of the form II crystals, allowing the bulk of a data set to be collected from a single crystal (Table 1). The form II crystals belong to space group $I222$ or $I2_12_12_1$, with post-refined unit-cell parameters of a = 37.11, b = 123.62, c = 164.65 Å. The volume of this unit cell (755338 Å^3) was consistent with the presence either one or two molecules in the asymmetric unit (V_M = 4.06 or 2.03 Å^3 Da^{-1}). These values correspond to 70 or 41% solvent, respectively. Given the very weak diffraction and the crystals' extreme sensitivity in the X-ray beam, the former possibility seemed more likely. Also, the self-rotation function calculated with these data failed to reveal any non-crystallographic

symmetry. A search for suitable heavy-atom derivatives is under way.

3.3. The form I structure cannot pack into the form II unit cell

Apo Δ(1–43)A-I adopts an unusual conformation in the form I crystals (Borhani *et al.*, 1997). The pseudo-continuous amphipathic α-helix, which is punctuated by kinks at regularly spaced proline residues, adopts a 'horseshoe' shape of dimensions 125 × 80 × 40 Å. Four molecules in the asymmetric unit associate *via* their hydrophobic faces to form an antiparallel four-helix bundle with an elliptical ring shape (135 × 90 × 60 Å). Can such an open molecular structure be packed into the form II unit cell?

Three facts constrain possible form II packing models. Firstly, the volume of the form II unit cell, the crystals' X-ray sensitivity and weak diffraction, and the lack of non-crystallographic symmetry all indicate that there is only one molecule in the asymmetric unit. Secondly, the unit cell is oddly proportioned: it is 4.4 times as long and 3.3 times as wide as it is deep. Thirdly, the highly symmetric unit cell can only accommodate molecules, especially one as large and open as the form I conformation of apo Δ(1–43)A-I, in certain locations of the unit cell without suffering from molecular overlaps.

There are two ways in which eight 'horseshoe'-shaped apo Δ(1–43)A-I molecules can be packed into the form II unit cell. (i) A monomer is centred at a point of 222 site-symmetry, (0,0,0), such that a tetramer is generated by application of *I*222 crystallographic symmetry. (This packing is not possible in $I2_12_12_1$, which has no sites with 222 symmetry.) Note that regularization of the pseudo-222-symmetry of the form I tetramer to 222 crystallographic symmetry significantly alters (shifts of 8–14 Å) most of the helix–helix registrations found in the form I crystal structure (Borhani *et al.*, 1997). (ii) The monomer is placed elsewhere in the unit cell, such that a tetramer is not formed.

3.3.1. Case 1: tetramers. The apo Δ(1–43)A-I tetramer has one twofold rotation axis (*Q*) and two pseudo-twofold axes (*P* and *R*; Borhani *et al.*, 1997). There are six ways to align these axes with the *I*222 crystallographic axes. We name them by whether the *P*, *Q* or *R* axes lie along *a*, *b*, or *c*. Each possibility was examined using molecular graphics (*O*; Jones *et al.*, 1991).

3.3.2. *PQR*, *PRQ*, *RPQ* and *RQP*. These orientations all suffer from multiple severe molecular interpenetrations along the short *a* axis (37.11 Å), as the *P* and *R* axes lie along the longest extents (135 and 90 Å, respectively) of the apo Δ(1–43)A-I tetramer. Also, there are very large gaps between molecules in the *bc* plane, leaving no interactions to stabilize the lattice.

3.3.3. *QPR* and *QRP*. As shown in Fig. 1 (*QRP* model), the saddle-shaped tetramers stack very tightly along *a* in the form II unit cell, as the thickness of the tetramer at any given point along its circumference is slightly less than the *a*-axis spacing. In the *bc* plane, the tetramers make many tight contacts along the periphery of the ring, as well as one serious molecular interpenetration. Minor adjustments in the dimension or shape of the tetramer just traded one set of bad contacts for another. The *QPR* model is also packed very tightly. The restricted packing in these form II models contrasts with the actual packing found in the form I crystals (Borhani *et al.*, 1997), in which the tetrameric rings are packed in a very open manner: the rings make only a few tenuous contacts with one another and large interconnected solvent channels run throughout the crystal in all three directions. How can the form II crystals (solvent content 70%) possess a much tighter molecular packing in all three directions (with solvent channels running in one direction only, along *a*) and yet diffract more poorly and be more sensitive to X-rays than the form I crystals (solvent content 75%; Leung *et al.*, 1999)?

3.3.4. Case 2: monomers. We examined several packing arrangements in which the monomer was shifted away from the point of 222 site symmetry. In all cases, we were unable to find a sensible arrangement which avoided serious molecular interpenetrations. The problem is that *I*222 contains twofold rotation axes interleaved with twofold screw axes. This crystallographic symmetry places strong constraints on where a molecule of the size and open shape of the apo Δ(1–43)A-I monomer can be placed.

3.3.5. Packing in $I2_12_12_1$. The situation in space group $I2_12_12_1$ was even worse. This space group is notoriously diffi-

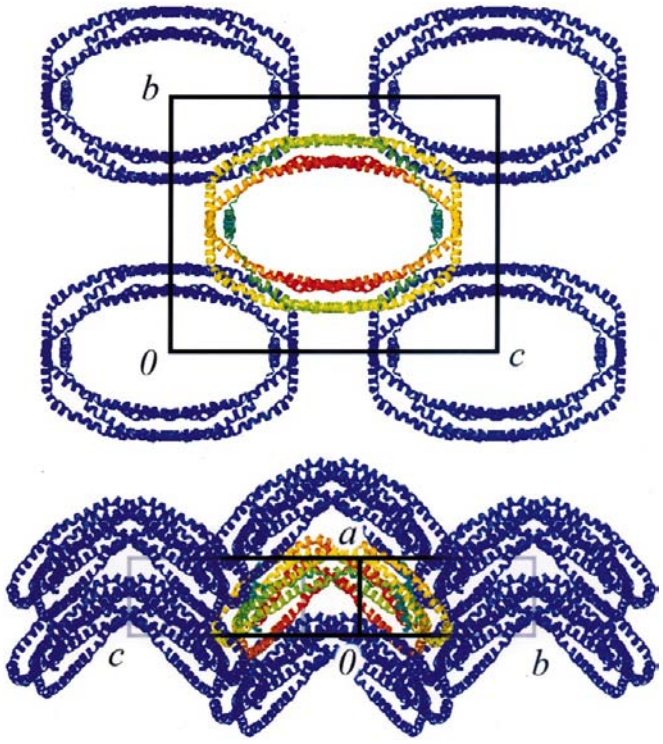

Figure 1
Molecular-packing model for the form I apo Δ(1–43)A-I helical 'horseshoe' in the form II crystals: the *QRP* model projected along the *a* axis (top) and the *bc* diagonal (bottom). Each chain of the central tetramer (generated from the monomer by *I*222 crystallographic symmetry) is coloured from red at the N-terminus to green at the C-terminus. The surrounding molecules are shown in blue. The unit cell is shown in black.

cult to pack (only five of 7442 crystal structure entries in the Protein Data Bank are in space group $I2_12_12_1$, whereas 176 entries are in space group $I222$). We examined several possible packing arrangements, including a *QPR*-oriented monomer centred on the twofold axis parallel to *a* at $(0,0,\frac{1}{4})$. This arrangement, as well as the others we examined, suffered from severe catenane-like molecular interpenetrations.

Therefore, we conclude that it is extremely unlikely for the open 'horseshoe'-shaped conformation of apo Δ(1–43)A-I, as found in the form I crystal structure, to exist in the form II crystals. This conclusion is also supported by the fact that no cross-rotation function solution was found to place the form I crystal structure monomer in the form II unit cell.

3.4. Likely conformation of apo Δ(1–43)A-I in the form II crystals

Compact (four-helix bundle, ∼75 × 30 × 30 Å) and extended (two-helix coiled-coil, ∼150 × 20 × 20 Å) models for the apo Δ(1–43)A-I monomer have been presented by Rogers *et al.* (Rogers, Roberts, Lebowitz, Datta *et al.*, 1998; Rogers, Roberts, Lebowitz, Engler *et al.*, 1998). We found that both models could easily be accommodated in the form II unit cell. The change from a ring-shaped molecule to either the compact bundle or the extended rod allowed much more flexibility in the placement of the molecules in the unit cell. In particular, when the extended monomer was placed in the *bc* plane of the *I*222 cell, with its two ends at fractional coordinates (0.65, 0.25, 0.05) and (0.60, 0.90, 0.80), an open arrangement of the molecules resulted, with appropriate intermolecular interactions and no bad contacts. This model is shown in Fig. 2. The molecule made angles of ∼90, 55 and 35° with the *a*, *b* and *c* axes, respectively. Also noteworthy was that the lattice contacts were relatively few in number, arising mostly from helices crossing at an angle of ∼65°. These limited contacts and the open nature of the resulting three-dimensional solvent channels are consistent with the weak diffraction of the form II apo Δ(1–43)A-I crystals.

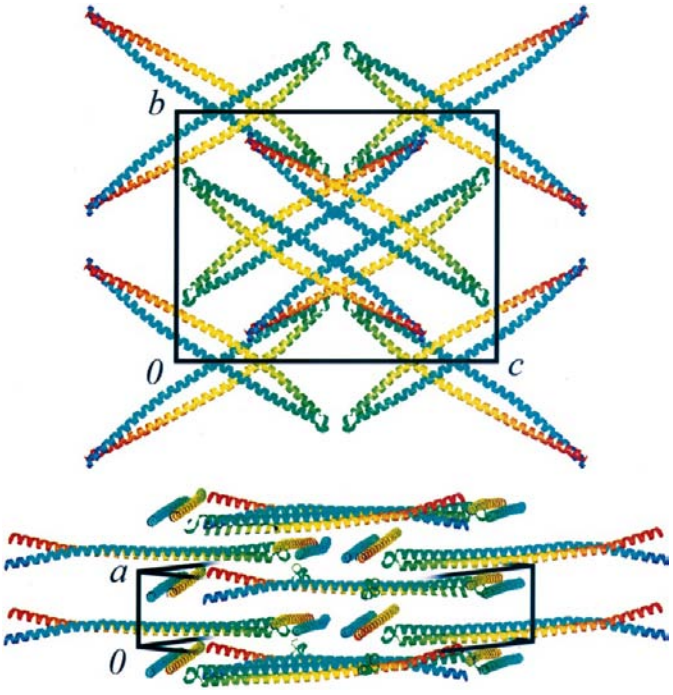

Figure 2
Molecular-packing model for an apo Δ(1–43)A-I extended helical rod in the form II crystals. Each monomer is coloured from red at the N-terminus to blue at the C-terminus. The molecular packing is shown projected along the *a* axis (top) and the *bc* diagonal (bottom).

4. Summary

We have crystallized the lipid-binding portion of human apolipoprotein A-I, the major protein component of high-density lipoprotein, in a new crystal form. These form II crystals grow at much lower ionic strength than the high-salt form I crystals. Using packing arguments, we have shown that the unusual open 'horseshoe'-like conformation of apo Δ(1-43)A-I found in the form I crystals (Borhani *et al.*, 1997) cannot be packed into the unit cell of the form II crystals. Rather, it is likely that monomeric apo Δ(1–43)A-I, as either a four-helix bundle or an extended helical rod, is the conformation adopted by apo A-I in the new crystals. Determination of the structure of apo Δ(1–43)A-I in the form II unit cell is likely to shed light on the conformational plasticity of this interesting and important protein.

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