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Structure Solution of C-Reactive Proteins: Molecular Replacement With a Twist

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Abstract

The pentameric structure of C-reactive proteins (CRP) has been derived by a combination of automated and manual molecular-replacement techniques. The method is generally applicable to other multimeric assemblies. The highly homologous human serum amyloid P component (hSAP) structure fails to provide a pentameric molecular-replacement solution for CRP. In the absence of a significant signal from an individual protomer, the hSAP structure has been manually modified in terms of protomer assembly to provide the true pentameric model of CRP. The CRP protomers are rotated or twisted by 14° about an axis, through the protomer centre, which is approximately perpendicular to the pentamer radius and the molecular fivefold axis. The results demonstrate clearly that protomers with very similar folds arising from high sequence homology need not necessarily be assembled together in the same way although the symmetry of the resulting oligomer may be maintained. In a curious twist the CRP structure which provided the general CRP model remains unsolved, while the model itself has so far provided the solution of two other CRP structures.

1. Introduction

Crystals of human C-reactive protein (hCRP) were first reported in 1947 (McCarty, 1947) but the structure has only recently been determined (Shrive *et al.*, 1996), along with that of rat CRP (TJG & co-workers, unpublished results). This is not only due to crystallization problems but also more recently to crystallographic problems involving protomer orientation, data completeness and 'false' pentameric solutions from both modelling (Srinivasan *et al.*, 1994) and molecular-replacement studies on these pentraxins. Usable isomorphous derivative crystals have not been reported for any CRP (DeLucas *et al.*, 1987; Myles, Bailey, Rule, Jones & Greenhough, 1990; Hopkins *et al.*, 1994) and the structure of the related pentraxin human serum amyloid P component (hSAP) (Emsley *et al.*, 1994) has been used as a model for molecular replacement (Rossmann, 1990). There is 51% strict homology between hCRP and hSAP and the protomers were expected to have the same fold and a pentameric arrangement. Protomers with very similar folds arising from sequence homology need not necessarily be assembled together in the same way although the symmetry of the resulting oligomer may be maintained. These differences in protomer assembly may arise from relatively few amino-acid differences, particularly if they are localized in regions involved in interprotomer contacts. We report here the molecular-replacement studies on the still unsolved Ca-depleted hCRP crystal structure, which led directly to a general pentameric model for CRP and the straightforward structure determination of both Ca-bound hCRP (Shrive *et al.*, 1996) and rat CRP.

C-reactive proteins are members of a conserved, phylogenetically ancient superfamily of oligomeric calcium-binding proteins exhibiting high sequence homologies (Gewurz, Zhang & Lint, 1995). Both CRP and the other major member of the pentraxin family (Osmand *et al.*, 1977), SAP, are characterized by a cyclic pentameric assembly of generally non-covalently associated subunits. Human CRP, first discovered in 1930 (Tillett & Francis, 1930) and the subject of intense clinical interest ever since (Pepys, 1995; Volanakis, 1993), is a trace plasma protein that is expressed rapidly and dramatically as part of the acute phase response to infection or injury (Abernethy & Avery, 1941; MacLeod & Avery, 1941). Ca-depleted hCRP was purified and crystallized in pentameric form (DeLucas *et al.*, 1987) and the determination of the fivefold directions within the crystal from self-rotation functions was thought to be straightforward for two tetragonal forms (Myles, Rule *et al.*, 1990). A reassessment of the data from both forms has subsequently revealed that crystals are almost always twinned and a re-examination of single crystals of one form has determined the space group as $C222_1$ although there is 'pathological' pseudosymmetry (Fitzgerald, 1992) present which has not yet been resolved and the possibility of lower point-group symmetry cannot be discounted entirely. Crystal data and data-set statistics are given in Table 1.

The *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) has been used throughout unless otherwise indicated. The spherical polar angles (ω,φ,κ) and Eulerian angles (α,β,γ) given are

Table 1. *Ca-depleted human C-reactive protein crystal data-set statistics*

Cell dimensions (Å)	$a = 145.28, b = 144.94, c = 306.78$	
Space group*	$C222_1$	
Resolution (Å)	3.0	
Matthews number† (Å^3 Da^{-1})	3.51	
Solvent content† (%)	65	
Data set‡	Initial	Final
No. of crystals	1	6
No. of images	50	74
No. of observations	119040	156777
No. of independent reflections	49367	52714
Redundancy	2.4	3.0
Completeness to 4.0 Å (%)	93.6	98.5
Completeness to 3.0 Å (%)	75.8	81.0
R_{merge}§ (%)	7.1	9.9

* Pseudosymmetry is present in the data and the possibility of lower point-group symmetry cannot be discounted entirely. Diffraction symmetry and systematic absences are, however, fully consistent with $C222_1$. † Based on two pentamers in the asymmetric unit; $Z = 2$. ‡ The initial data set was collected from one crystal, on station 9.6 ($\lambda = 0.882$ Å) at the CCLRC Daresbury SRS, using a large MAR image plate. Further data collected on station 7.2 ($\lambda = 1.488$ Å, small MAR) at the SRS were later added to fill in the systematically missing 'blind region' data, giving the final data set. § Defined as $\sum |I(h)_i - \langle I(h)\rangle| / \sum I(h)_i$ summed over all observations i.

according to *CCP*4 convention and although a single α,β,γ rotation may often be given it is representative not only of the crystallographic symmetry-related rotations but also of the non-crystallographic symmetry where pentamer rotations are concerned, that is, γ is $\gamma + n72°$ where $n = 0$–4. The pentameric search model, hSAP (Emsley *et al.*, 1994), was oriented with its fivefold axis along z (orthorhombic c) and with the centre of mass of a protomer (designated protomer 5) at $(x,0,z)$ for the molecular-replacement studies. An α,β solution for the fivefold direction in the cross-rotation function will therefore be numerically equivalent to φ,ω in the self-rotations fuction. The identity matrix is shown as $[I]$.

2. Determination of fivefold direction

The pentameric nature of hCRP, a highly conserved entity of identical protomers, is readily observed (Osmand *et al.*, 1977) and self-rotation function studies of the new orthorhombic data, using the initial data set, suggested fivefold directions of $\omega = 0$ and $\varphi = 90$, $\omega = 58°$. These solutions were not outstanding but no other significant fivefold peaks were observed. The initial data set was 94% complete to 4.0 Å with the missing data concentrated in a cusp around the c^* ($00l$) direction; the blind region for the crystal mounted with c^* along the rotation axis. When data completeness in the rotation function was increased from 94 to 99% (the final data set), the $\kappa = 72$ and 144° sections showed a single, consistent outstanding solution at $\varphi = 90$, $\omega = 58°$ (see Fig. 1). Solvent content, Matthews number (Matthews, 1968) and packing considerations suggest that there is more than one pentamer per asymmetric unit, the most probable number being two (see Table 1). The self-rotation function results suggest that the hCRP fivefold direction is the same for each independent pentamer.

Cross-rotation functions were carried out with the pentameric hSAP search model and structure factors were calculated in $P1$ with a unit cell large enough to exclude intermolecular vectors. A wide variety of cross-rotation function studies [*ALMN*, *AMoRe*, *GLRF* (Tong, 1993), *X-PLOR* (Brünger, 1992)] gave $\alpha = 90$, $\beta = 58°$, confirming the direction of the hCRP fivefolds in the orthorhombic crystals, although the relative rotations (γ) of the model were not clearly defined with several equally convincing potential solutions present. A series of cross-rotation functions, with a Patterson inner radius of 8 Å and an outer radius ranging from 15 to 35 Å, in 5 Å steps, for each of the resolution ranges 14–8, 14–5, 14–4, 8–5 and 8–4 Å, were carried out. Clustering of the results suggested $\alpha = 90$, $\beta = 62$, $\gamma = 30°$ and/or $\alpha = 90$, $\beta = 63$, $\gamma = 19°$ as the most probable solutions (Holden, 1996). None of the pentameric orientations provided by the cross rotation however gave either a convincing Patterson correlation (PC) refinement in *X-PLOR* or a solution in translation-function studies.

Very low resolution cross-rotation functions were calculated in an attempt to ascertain the approximate relative rotations (γ's) required. Two separate solutions, one again in the region of $\alpha = 90$, $\beta = 64$, $\gamma = 30°$ and the other around $\alpha = 95$, $\beta = 66$, $\gamma = 5°$, often occurred, though not necessarily simultaneously nor in every case. Neither possibility provided any progress towards the structure solution. Modifying the search model in a variety of ways, for example, backbone only or with various hSAP/hCRP non-homologous regions omitted, gave no improvement in any of the results.

3. Protomer studies

Exhaustive attempts (using *AMoRe*, *GLRF*, *X-PLOR*) were made to find the Ca-depleted hCRP molecular-replacement solution using a single hSAP protomer, both intact and also with various hSAP/hCRP non-homologous regions omitted from the model (Holden, 1996). The calculations were carried out using the same resolution and integration radii (25 runs) as in the pentamer studies, and using the modified or unmodified search protomer 5 selected from the hSAP model oriented with the fivefold along z (c). Large numbers of possible solutions with little or no discrimination were produced. To reduce the problem the solutions were investigated to find those corresponding to components of the same pentamer oriented about the assumed fivefold direction. If the coordinates $\mathbf{p}$ of the search protomer are rotated to $\mathbf{q}_i$ by a rotation $[R_i]$ then,

$$\mathbf{q}_i = [R_i]\mathbf{p}. \quad (1)$$

If two protomer rotations $[R_i]$ and $[R_j]$, resulting in coordinates $\mathbf{q}_i$ and $\mathbf{q}_j$, respectively, are solutions to the protomer rotation function then,

$$[S] = [R_j][R_i]^{-1}, \quad (2)$$

will rotate $\mathbf{q}_i$ onto $\mathbf{q}_j$ and $[S]$ should correspond to $\varphi = 90$, $\omega = 58$, $\kappa = n72°$, the probable fivefold direction of the hCRP pentamers.

While hundreds of protomer rotation functions were calculated using a variety of software and models, the results from the fast rotation function option of *GLRF* appeared to be the most promising and those from the complete protomer search model are described here. The number of possible solutions (the top 40 peaks from each of the 25 runs) was reduced first by selecting only those which were approximately related to at least one other solution by a $n72°$ rotation about $\varphi = 90$, $\omega = 58°$ ($[S]$), and then, since many were similar, by clustering together orientations within 6° bands of α, β and γ (reducing the initial 1000 solutions to 246 and then 115). Solutions not contributing to any cluster were then

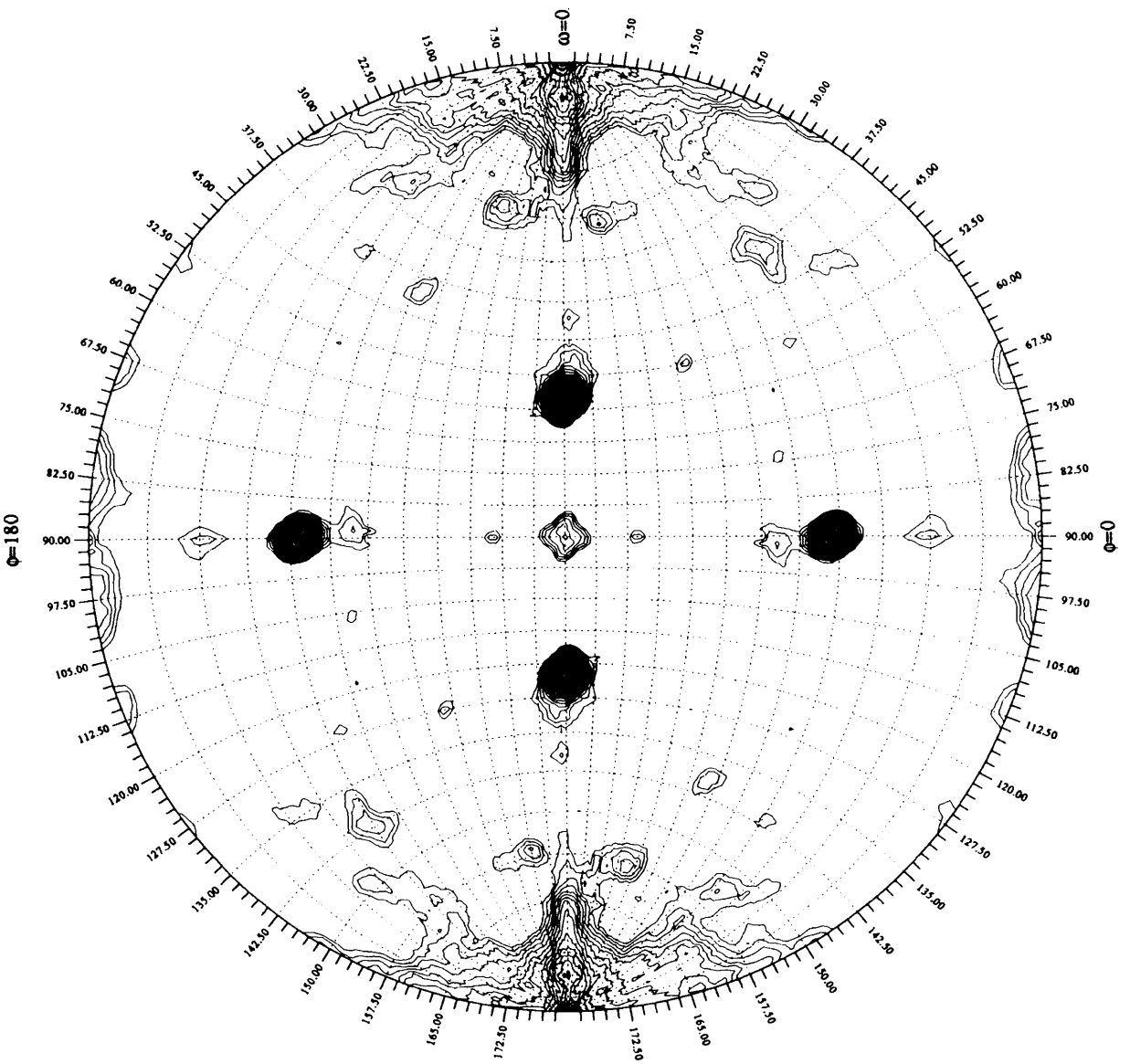


Fig. 1. Stereographic projection of the $\kappa = 72°$ section of the Ca-depleted hCRP $C222_1$ self-rotation function. The rotation function was calculated using *GLRF* with origin removed *E*'s, using the 8–4 Å data, Patterson integration radii of 35–12 Å and φ,ω steps of 1.5°. The centre of the plot corresponds to $\varphi = 90$, $\omega = 90°$. The fivefold solution peak at $\varphi = 90$, $\omega = 58°$ is seven standard deviations above background. Peaks at $\varphi = 90$, $\omega = 58°$ are present on both the $\kappa = 72°$ and $\kappa = 144°$ sections. The peak at $\varphi = 32°$, $\omega = 90°$ is a symmetry equivalent of the $\varphi = 90$, $\omega = 58$, $\kappa = 144°$ peak. There is no peak at $\varphi = 32$, $\omega = 90$, $\kappa = 144°$.

Table 2. *Clustered protomer rotation-function solutions [R] involved in relationships consistent with a pentamer with the expected fivefold direction*

	$[R_i]$				$[R_j]$			$[S] = [R_j][R_i]^{-1}$			PC refined $[R_i]$			PC
	α	β	γ		α	β	γ	φ	ω	κ	α	β	γ	
1	147.6	73.0	355.8	27	43.6	90.0	251.7	89.0	54.9	212.8	150.7	76.3	354.4	0.0195
2	143.4	67.0	71.7	24	57.2	90.0	323.4	91.9	57.6	216.0	140.1	65.4	74.6	0.0175
3	142.0	69.0	11.9	25	54.8	90.0	265.8	90.5	57.1	218.2	144.0	66.8	9.8	0.0147
4	133.6	74.0	185.6	26	52.0	79.0	71.7	91.1	58.3	213.1	132.7	78.5	184.7	0.0211
5	129.4	90.0	178.6	28	36.6	75.0	71.7	88.6	58.0	218.7	131.4	98.3	174.5	0.0236
				29	34.7	72.0	72.2	88.8	56.9	216.0				
6	116.7	67.0	226.4	22	66.1	43.0	40.8	90.7	58.7	146.5	116.0	67.5	227.4	0.0133
				23	64.1	40.0	40.5	89.5	57.6	144.1				
7	106.9	63.3	151.0	17	77.8	66.0	19.7	91.7	59.7	215.0	103.6	63.3	153.7	0.0181
				19	74.5	56.0	25.3	92.9	54.0	216.3				
8	105.5	43.0	1.4	11	95.6	76.0	226.9	92.7	58.2	218.4	107.9	41.8	359.4	0.0178
9	103.5	57.2	222.8	12	95.6	71.0	150.5	89.2	59.9	282.9	103.4	56.9	219.6	0.0211
				19	74.5	56.0	25.3	88.9	59.4	147.1				
10	99.8	69.0	201.1	16	78.8	73.0	140.6	85.7	56.3	290.0	100.7	70.2	202.7	0.0265
				21	70.3	54.0	75.9	89.5	56.1	218.9				
11	95.6	76.0	226.9	8	105.5	43.0	1.4	92.7	58.2	141.6	94.4	76.2	225.7	0.0248
12	95.6	71.0	150.5	9	103.5	57.2	222.8	89.2	59.9	77.1	96.0	71.3	153.4	0.0246
13	94.2	44.0	63.3	15	83.0	69.0	210.9	92.9	58.0	142.3	94.3	43.8	62.6	0.0145
14	90.5	71.3	207.6	18	77.8	65.3	140.6	89.0	60.2	287.2	91.8	73.3	209.7	0.0334
				20	73.1	61.5	145.5	90.6	55.1	288.7				
15	83.0	69.0	210.9	13	94.2	44.0	63.3	92.9	58.0	217.7	89.7	77.6	211.1	0.0216
16	78.8	73.0	140.6	10	99.8	69.0	201.1	85.7	56.3	70.0	78.8	72.0	140.6	0.0262
				21	70.3	54.0	75.9	90.8	59.0	288.8				
17	77.8	66.0	19.7	7	106.9	63.3	151.0	91.7	59.7	145.0	77.0	66.1	20.4	0.0229
18	77.8	65.3	140.6	14	90.5	71.3	207.6	89.0	60.2	72.8	80.0	71.0	139.7	0.0229
19	74.5	56.0	25.3	7	106.9	63.3	151.0	92.9	54.0	143.7	75.7	58.5	26.5	0.0210
				9	103.5	57.2	222.8	88.9	59.4	212.9				
20	73.1	61.5	145.5	14	90.5	71.3	207.6	90.6	55.1	71.3	72.2	61.5	146.3	0.0206
21	69.6	55.5	75.2	10	99.8	69.0	201.1	88.6	56.7	141.7	66.5	54.1	78.1	0.0180
				16	78.8	73.0	140.6	88.9	59.1	71.8				
22	66.1	43.0	40.8	6	116.7	67.0	226.4	90.7	58.7	213.5	69.8	44.0	39.2	0.0220
23	64.1	40.0	40.5	6	116.7	67.0	226.4	89.5	57.6	215.9	69.3	41.2	38.3	0.0185
24	57.2	90.0	323.4	2	143.4	67.0	71.7	91.9	57.6	144.0	58.5	91.4	322.8	0.0120
25	54.8	90.0	265.8	3	142.0	69.0	11.9	90.5	57.1	141.8	53.6	91.1	260.0	0.0142
26	52.0	79.0	71.7	4	133.6	74.0	185.6	91.1	58.3	146.9	51.2	78.7	71.9	0.0131
27	43.6	90.0	251.7	1	147.6	73.0	355.8	89.0	54.9	147.2	40.7	89.3	250.4	0.0107
28	36.6	75.0	71.7	5	129.4	90.0	178.6	88.6	58.0	141.3	33.3	69.2	65.8	0.0175
29	34.7	72.0	72.2	5	129.4	90.0	178.6	88.8	56.9	144.0	36.8	74.2	69.5	0.0156

removed and of the remaining 47 clustered orientations 29 were still involved in relationships where $[S]$ (2) corresponded to approximately $\varphi = 90$, $\omega = 58$, $\kappa = n72°$. These solutions $[R_i]$, $[R_j]$ are shown in Table 2 along with the resulting $[S]$. Only two sets of three related orientations were found, the first containing solutions 10, 16 and 21; the second containing either 9, 12 and 19 or 7, 9 and 19. Both of these sets may be solutions if the relative rotation, γ, is different for each independent pentamer, but only the first is fully internally consistent with all three inter-relationships present. Translation functions (TFFC) using $[R_i]$ were generally flat with little discrimination. The only $[R_i]$ which gave a peak of any kind not placing the protomer on a twofold symmetry element was solution 10. PC refinement in *X-PLOR* did appear to give some discrimination (see Table 2) but again only solution 10 gave a possible translation (the tenth highest peak).

Even with a set of five consistent protomer rotations, a solution still depends on finding at least two correct protomer translations in each independent pentamer. An infinite number of pentamers can be produced from a set of five protomer orientations with no knowledge of which might be correct without information from the translation function. For two independent pentamers a protomer represents only 1/10 of the asymmetric unit and both the rotation and translation signal must necessarily be weak. It may be beneficial to use the direct rotation function (DeLano & Brünger, 1995) to find the individual protomer rotations, since this has been observed to give a much better signal-to-noise ratio for partial-structure search models (Berchtold *et al.*, 1993; Gewirth & Sigler, 1995), although the inter-protomer relations would still have to be found. The locked rotation function in *GLRF* provides an automated protomer orientation search for the detection of non-crystallographic symmetry about a chosen direction, although protomer–protomer contacts are necessarily excluded and, in this case, the fivefold direction must be assumed from the self-rotation function. The locked

rotation function was used in conjunction with the individual protomer studies, but no convincing results were obtained. The correct solution, in retrospect, was not present in the top 40 peaks from a full asymmetric unit search using 12–4.5 Å data, a step size of 3° and a fivefold local symmetry orientation equivalent to $\varphi = 90$, $\omega = 58°$.

With a single possibility for the ten protomer rotation/translations present, and with no indication that the translation might be correct, the protomer work was abandoned in favour of the rebuilt pentamer strategy detailed below. This has the advantages of not relying on any assumptions regarding the fivefold direction(s), and of a pentameric search model.

4. Determining a new model

Concomitant with the protomer studies, a 'brute force' approach to solving the molecular-replacement problem, involving reorienting a protomer and rebuilding a pentamer, was also carried out. The strategy involved taking a single hSAP protomer from the search model (protomer 5), translating it to the origin, rotating it by a chosen amount about any chosen direction, then translating it back to its original position and producing the pentamer by cyclic 72° rotations. Thus, if the protomer coordinates **p** are transformed to **q** by this operation then,

$$\mathbf{q} = \mathbf{x}_2 + [\Phi](\mathbf{x}_1 + \mathbf{p}), \tag{3}$$

where $\mathbf{x}_1$ is the translation to the origin (pentamer radius of 31.8 Å), $[\Phi]$ is the rotation κ about the direction defined by polar angles, ω,φ nds x_2 is the translation in the $-\mathbf{x}_1$ direction (see Fig. 2). If $\mathbf{x}_2 = -\mathbf{x}_1$ then the protomer is returned to its original position while $\mathbf{x}_2 \neq \mathbf{x}_1$ allows variation in the pentamer radius to be investigated. Although the problem is described fully by (3), in order to account for different pentamer builds from a particular protomer orientation, perhaps for a promising $[\Phi]$, the translation $\mathbf{x}_2$ may be replaced by $\mathbf{x}_3$ (see Fig. 2), with an additional rotation $[K]$ about $\omega = 0°$ returning the protomer centre to its original position. Hence,

$$\mathbf{q} = [K]\{\mathbf{x}_3 + [\Phi](\mathbf{x}_1 + \mathbf{p})\}, \tag{4}$$

If $|\mathbf{x}_3| = |\mathbf{x}_2|$ then $\mathbf{x}_2 = [K]\mathbf{x}_3$ giving,

$$\mathbf{q} = \mathbf{x}_2 + [K][\Phi](\mathbf{x}_1 + \mathbf{p}), \tag{5}$$

and the variation in protomer build may be accounted for, by comparison with (3), by an additional rotation $[K]$ about $\omega = 0°$ prior to the final shift $\mathbf{x}_2$. If (5), with both $[\Phi]$ and $[K]$ varying, were to be used for the general search rather than (3), then the value of ω in $[\Phi]$ may be restricted to $\omega = 90°$. The pentamer is rebuilt by cyclic 72° rotations of **q** about $\omega = 0°$. Cross-rotation functions were carried out using the rebuilt pentamer search model and the program *ALMN*. The top sets of peaks, and the top set which corresponded approximately to the probable fivefold direction $\alpha = 90$, $\beta = 58°$, were then listed according to root-mean-square deviation from the mean (r.m.s.). This was performed for each new pentamer.

As an initial step, a rotation of 15° about each of the major (432) axes of a cube was applied to the protomer at the origin. Thus, in (5) $\mathbf{x}_2 = -\mathbf{x}_1$, $[K]$ is the identity and $[\Phi]$ contains values of $\omega,\varphi,15$ corresponding to a 15°

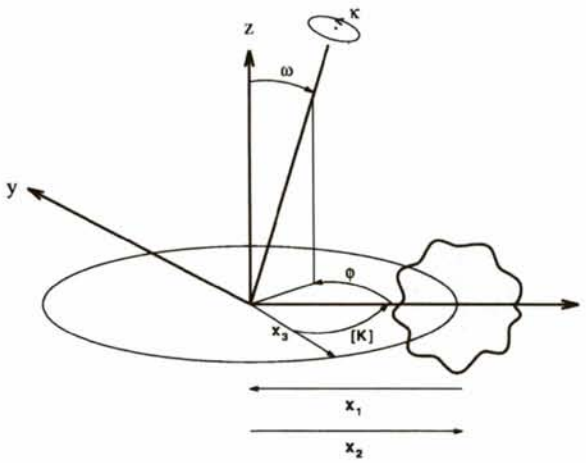


Fig. 2. Generation of the rebuilt pentameric search model from hSAP protomer 5 (shaded). The protomer is initially positioned at $y = 0$ in the hSAP pentamer oriented with the fivefold along z. The rebuilt pentamer is generated by translating protomer 5 to the origin by $\mathbf{x}_1$, rotating by κ about the direction ω,φ and translating back by $\mathbf{x}_2$. Variations in pentamer build from the same protomer orientation $[\Phi]$ (ω,φ,κ) may be accounted for by an additional rotation $[K]$ about $\omega = 0°$ prior to the final shift $\mathbf{x}_2$. The pentamer is then rebuilt by cyclic 72° rotations about $\omega = 0°$.

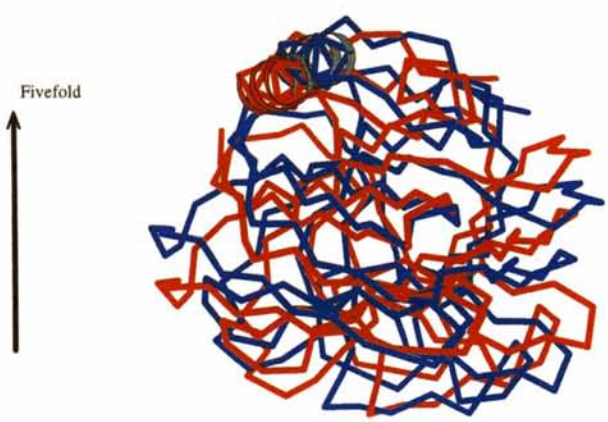


Fig. 3. Protomer 5 from the hSAP search model in the original hSAP pentamer configuration (blue) and in the derived model for CRP (red) (*MOLSCRIPT*, Kraulis, 1991). The pentameric fivefold is oriented along z (**c**). A 14° rotation about a circumferential axis (into paper) approximately through the centre of the protomer superposes the original hSAP configuration onto the CRP configuration. This rotation is sufficient to prevent a molecular replacement solution for CRP using the hSAP pentameric structure.

rotation about the chosen cubic axes. This magnitude of rotation angle was chosen as there was not any reason to believe that the protomer rotation with respect to hSAP would be large. A rebuilt pentamer using the correct protomer orientation would be expected to give a peak in the cross-rotation function whether the pentamer was built correctly or not, with an even more significant signal expected when the protomers were correctly assembled together. At a resolution range of 10–6 Å and an integration radius of 30–5 Å, the initial rotations showed large discrimination in r.m.s. level generally, and for the top 'correct' fivefold direction in each case particularly, although not all orientations produced the probable fivefold direction within the top 40 peaks. One protomer orientation region was seen to give much larger significant peaks than the rest, with a level of 4.5 r.m.s. compared to levels of 2.3–3.8 r.m.s. for other regions. These large peaks corresponded to the probable fivefold direction, $\alpha = 90$, $\beta = 58°$. This region was investigated in finer detail using variable [Φ] and pentamer radius ($\mathbf{x}_2$) and a variety of resolution ranges and integration radii. The search converged, based on the r.m.s. level, to $[K] = [I]$, $\mathbf{x}_2 = -\mathbf{x}_1$ and $\omega = 88$, $\varphi = 95°$, $\kappa = -14°$ ($[\Phi_1]$); a protomer rotation of 14° about a direction which was approximately tangential to the circumference of the pentamer (see Fig. 3). This can be envisaged as holding the hSAP protomer at its pentameric contacts and pivoting about these by 14°, thereby maintaining the interprotomer contacts, some of which were believed to be conserved based on the homology with hSAP (Srinivasan *et al.*, 1994). The cross-rotation function solution was $\alpha = 87$, $\beta = 58$, $\gamma = -5°$ ($[M_1]$). It should be noted that this protomer orientation did indeed give a significant signal over a broad area, such that maintaining the protomer orientation $[\Phi_1]$ but rebuilding the pentamer in different ways {by varying $[K]$ in (5)} consistently gave a large signal at the expected α,β with the first pentamer build giving one of the highest (see Fig. 4). Translation functions (TFFC) on the optimized, PC-refined (*X-PLOR*), new pentameric build ($[\Phi_1]$, $[K] = [I]$, $\mathbf{x}_2 = -\mathbf{x}_1$) rotated by $[M_1]$ gave an outstanding (38 r.m.s.) solution peak (see Fig. 5) while rebuilding the pentamer in different ways with this protomer orientation did not. This clearly emphasizes the importance of establishing the correct protomer–protomer contacts in translation-function studies. The fivefold direction of the refined solution was $\alpha = 90.0$, $\beta = 56.8$, $\gamma = -8.2°$ ($[M_2]$) for the pentamer refinement and $\alpha = 89.6$, $\beta = 57.1$, $\gamma = -8.2°$ ($[M_3]$) for the protomer refinement. Each of the protomers in the PC-refined solution gave an individual translation consistent with the pentameric solution.

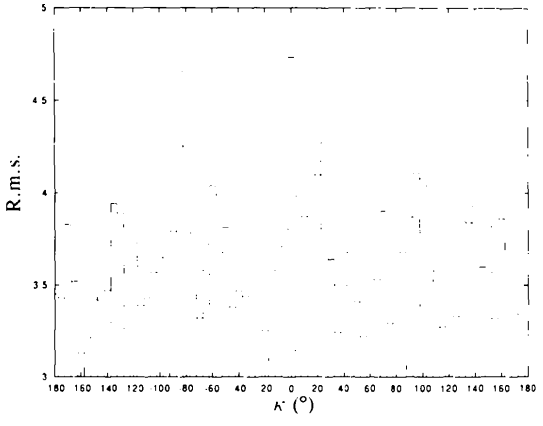


Fig. 4. Variation in rotation function r.m.s. level with pentamer build $[K]$ for the protomer solution $[\Phi_1]$. $[K]$ represents a rotation of κ about the $\omega = 0°$ direction. Only the $\kappa = 0°$ solution gives a translation-function solution.

The rebuilt pentamer based on one protomer orientation produces a single rotation-function solution and a single outstanding translation-function solution but there are (most probably) two independent pentamers in the asymmetric unit. Although the fivefold direction was believed to be approximately the same for both, there was no reason to suspect that the protomer orientation within the pentamer should be different, nor that the rotation of the pentamer (γ) about the fivefold should be the same, in both. Protomer orientations with [Φ] ranging throughout polar space were investigated, with κ rotations varying from 5 to 20° and in finer detail than the initial search, to find other significant orientations. Several additional orientations were selected as candidates and optimized, first on [Φ] and then on $[K]$, but translation functions did not produce any further solutions, leading to the conclusion that both independent pentamers were oriented with the same α, β and γ.

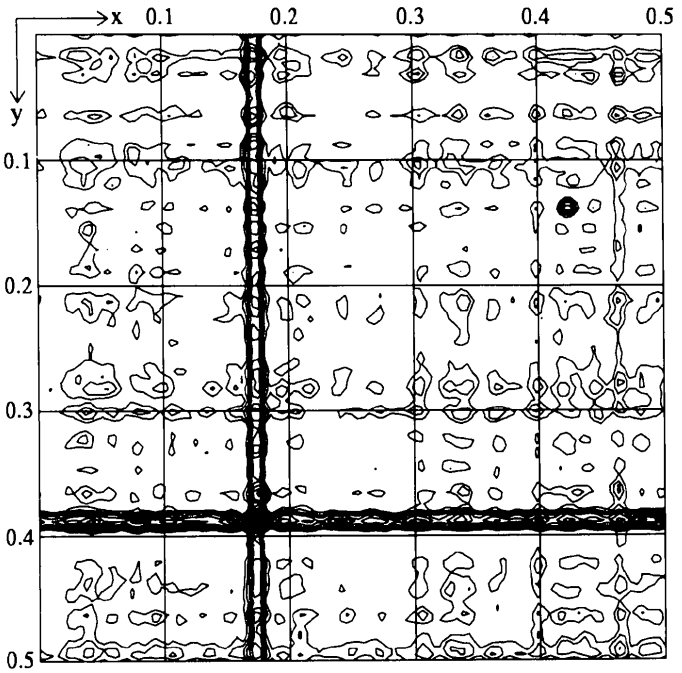
Investigation of the translation function (see Fig. 5) for the rebuilt pentamer solution has led to the conclusion that there is pseudosymmetry present in the Ca-depleted hCRP crystals. A consistent interpretation of the rotation and translation function results in terms of two independent pentamers in $C222_1$ related by a non-crystallographic twofold axis at $y = 1/4$ parallel to the crystallographic twofold along x has been derived (TJG & AKS, unpublished results), although inspection of the native Patterson gives no strong indication of the resulting non-crystallographic translational symmetry. The structure of this crystal form still eludes us.

5. Investigation of solution

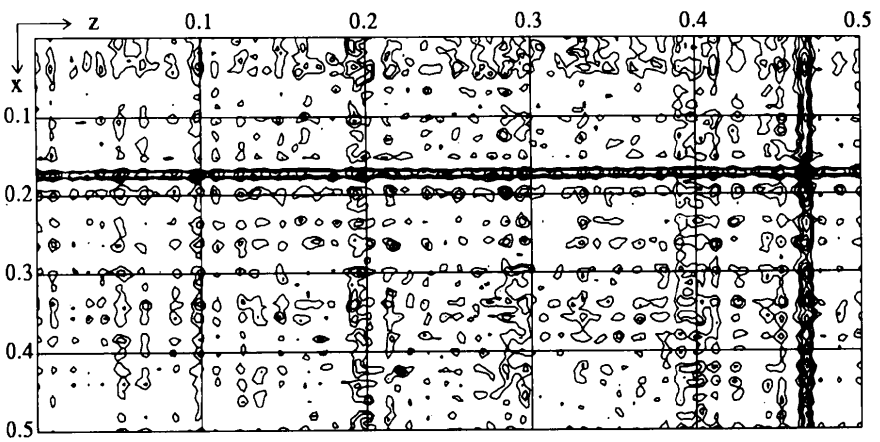
The rebuilt pentamer solution was compared with the results from the protomer studies. If the true protomer rotation within the hCRP pentamer relative to hSAP is $[K][\Phi_1]$ from the rebuilt pentamer studies, and the solution to the rotation function for this re-oriented protomer is $[M_1]$ then,

$$[R1_i] = [M_1][C_i][K][\Phi_1], \qquad (6)$$

are the rotations required to transform the original hSAP protomer 5 to the hCRP orientation, where $[C_i]$ represents the cyclic 72° rotations which build the pentamer. For the unrefined solution, $[M_1]$ corresponds to $\alpha = 87$, $\beta = 58$, $\gamma = -5°$, $[\Phi_1]$ to $\omega = 88$, $\varphi = 95$, $\kappa = -14°$ and $[K] = [I]$, giving the values of $[R1_i]$ shown in Table 3. These values superpose the unrotated search protomer 5 onto each protomer of the hCRP solution. Also given in Table 3 are the values of $[R2_i]$ and $[M_2]$ corresponding to the PC refinement of the pentamer from $[R1_i]$, and of $[R3_i]$ and $[M_3]$ corresponding to the further protomer PC refinement of $[R2_i]$. Comparison with the single protomer rotation-function results $[R]$ given in Table 2 shows that the triplet of protomer orientations 10, 16 and 21 correspond approximately to $[R_3]$, $[R_2]$ and $[R_1]$, respectively, in the solution. Reinspection of the complete list of protomer orientations failed to reveal a solution corresponding to either $[R_4]$ or $[R_5]$. The translation found for protomer orientation 10 (100.7, 70.2, 202.7°) also corresponded closely to that for $[R3_3]$. Rebuilding the pentamer from orientation 10 according to (5) may provide the solution, but assumptions would have to be made regarding the fivefold direction (to determine $[\Phi]$), while $[K]$ would have to be varied to determine the pentamer build. This would also be the case for protomer orientations determined by any other procedure, including the direct rotation function (DeLano & Brünger, 1995), if protomer translations



(*a*)



(*b*)

Fig. 5. Translation function (TFFC) map (calculated using data to 3.0 Å) for the Patterson correlation refined solution $[M_3]$ from the rebuilt pentamer solution. The outstanding 38r.m.s. peak is at fractional coordinates $x = 0.17$, $y = 0.39$, $z = 0.47$. None of the other pentameric rebuilds produced a translation solution. Both the outstanding height of the peak and the absence of a solution for the second independent pentamer may be explained in terms of a pseudo twofold parallel to x at $y = 1/4$ although no strong evidence of this is present in the native Patterson (see text). (*a*) xy section at $z = 0.47$ (*b*) zx section at $y = 0.39$.

Table 3. *Rotation solutions mapping the hSAP search protomer 5 onto each protomer i in the pentameric hCRP solution*

The unrefined rotations correspond to the pentamer solution produced by manually rebuilding the pentamer and optimizing the signal in the cross-rotation function. Pentamer refined refers to this solution PC refined as a pentameric rigid body, while protomer refined refers to further PC refinement of all five individual protomers. [*M*] is the corresponding fivefold direction in each case.

	Unrefined			Pentamer refined			Protomer refined		
	α	β	γ	α	β	γ	α	β	γ
i		$[R1_i]$			$[R2_i]$			$[R3_i]$	
1	70.7	54.7	75.6	73.6	52.7	72.8	69.2	55.2	73.9
2	78.2	69.6	142.4	80.5	67.9	139.7	81.9	72.8	138.8
3	95.7	69.6	206.7	98.0	68.8	203.6	100.7	68.5	201.0
4	103.4	54.8	273.4	106.8	54.4	269.8	107.3	53.9	269.7
5	87.1	44.0	354.5	91.3	42.8	350.5	87.0	43.4	351.7
		$[M_1]$			$[M_2]$			$[M_3]$	
	87	58	−5	90.0	56.8	−8.2	89.6	57.1	−8.2

could not be established. While use of *AMoRe* itself failed to provide any consistent protomer orientations, it may be that the use of the *GLRF* derived rotation and the *TFFC* translation for protomer solution 10 would have provided further solutions in a stepwise procedure.

Model-to-model cross-rotation functions give insight into why the initial studies failed to provide the pentameric hSAP to CRP rotation. The rebuilt pentamer aligned with the fivefold along z (**c**) was rotated by $[M_3]$ ($\alpha = 89.6$, $\beta = 57.1$, $\gamma = -8.2°$) and structure factors calculated in $P1$ from this model. Cross-rotation functions with this pentamer and the original, unrotated hSAP structure (Emsley *et al.*, 1994), again in $P1$, did not give $[M_3]$ and were, as observed in the initial study, often inconsistent and inconclusive. The top solutions generally corresponded to components of the true protomer rotations $[R3_i]$ (see Table 3). The only peaks corresponding to the expected fivefold direction were $\alpha = 91$, $\beta = 57$, $\gamma = 37°$ and $\alpha = 89$, $\beta = 56$, $\gamma = 19°$ for the 10–6 Å data, and $\alpha = 89$, $\beta = 56$, $\gamma = 30°$ and $\alpha = 90$, $\beta = 57$, $\gamma = 20°$ for the 12–4 Å data. These false solutions correspond to the most probable solutions found from the initial cross-rotation function studies (*vide infra*). As a direct result of the different protomer orientation in CRP as compared to hSAP, the correct orientation of the hCRP pentamer is not found from the cross-rotation function using the hSAP pentameric search model.

6. Conclusions

Apart from all the difficulties which may occur in determining non-crystallographic symmetry directions using marginally incomplete data, these CRP crystals have illustrated that there may also be many other factors to consider. Systematically missing data, even at the 5% level reported here, can affect the self-rotation function significantly. Only the 99% complete data set in the self-rotation function provided a fivefold direction in which there was sufficient confidence to proceed with an interpretation of the massive number of possible solutions.

Protomers of hSAP and hCRP are highly homologous with very similar folds and both have cyclic pentameric aggregations. Despite these similarities and the conservation of two interprotomer salt bridges (Shrive *et al.*, 1996) there is a marked difference in protomer assembly and, due to insufficient signal from an individual protomer, it has been necessary to manually rebuild a pentameric assembly to provide the correct CRP model. High homology and tertiary structure similarity do not necessarily imply that correct prediction of the molecular structure by modelling is readily achievable, as evidenced by the work of Srinivasan *et al.* (1994) where the protomer orientation has not been accounted for. The different subunit disposition within the pentamer with respect to hSAP not only prevents a quick and simple solution to the molecular-replacement problem, but also gives time-consuming misleading answers in the cross-rotation function.

Although it has not been possible at this stage to solve the structure of the Ca-depleted form of hCRP, this new pentameric model has allowed the straightforward structure determination of rat CRP (TJG and co-workers, unpublished results) and Ca-bound hCRP (Shrive *et al.*, 1996) which has two independent pentamers in the asymmetric unit. In addition to providing a true pentameric model of CRP structures in general, the methods outlined here are generally applicable to other oligomeric cases where the single protomer signal is weak. This procedure is particularly powerful in these cases and also provides a rapid way forward when a single, well established orientation is combined with knowledge of molecular symmetry and aggregation. Application of (5), followed by a molecular rebuild based on the symmetry and number of protomers present rather than the cyclic 72° used here, should greatly facilitate the solution of difficult molecular-replacement problems involving oligomeric assemblies.

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