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A study of the denaturation of human C-reactive protein in the presence of calcium ions and glycero-phosphorylcholine

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Abstract

Differential scanning calorimetry, fourier transform infrared spectroscopy and surface plasmon resonance spectrometry have been used to study the thermal properties of C-reactive protein in its ligand-bound and ligand-free forms. Clear denaturation endotherms were detected during calorimeter heating scans. Upon binding either or both its ligands, Ca^{2+} and glycero-phosphorylcholine, the denaturation temperature of the protein and the enthalpy change on denaturation were increased. Marked changes in the infrared spectrum of the protein were seen when it was heated. The main absorption peaks in the spectrum, at 1651 and 1643 cm^{-1} were replaced by a single peak at 1628 cm^{-1}. There was an isosbestic point at 1630 cm^{-1}. Comparison with the calorimetric data indicated that the changes in spectra were caused by protein denaturation. The spectra, like the calorimetric data, showed that the presence of glycero-phosphorylcholine raised the denaturation temperature of the protein. However, the presence of calcium ions had little effect on the spectra. This suggests that unlike glycero-phosphorylcholine, calcium binds to C-reactive protein in such a way that hydrogen bonding in the polypeptide backbone is not altered. Using surface plasmon resonance spectrometry the adsorption of C-reactive protein onto self-assembled phosphorylcholine monolayers was measured in the presence of the protein's ligands before and after its denaturation. Monolayers were formed by reacting a phosphorylcholine alkyl thiol with the surface of silvered microscope slides. It was possible to demonstrate a specific interaction between C-reactive protein and the phosphorylcholine surface and show that this interaction did not take place after the protein had been denatured.

Keywords: C-reactive protein; Glycero-phosphorylcholine; Ligands

1. Introduction

The initial response of the body to infection, inflammation or tissue injury is referred to as acute phase response [1]. The characteristics of this response include the rapid increase in concentration of specific serum proteins collectively called acute phase reactants [2]. C-reactive protein is the prototypic acute phase reactant, originally discovered in the serum of patients with pneumonia, as a precipitin for the C-polysaccharide of the pneumococcal cell wall [3]. During the course of infection the amount of circulating C-reactive protein was found to increase to 0.1 mg cm^{-1}, 1000-fold or more over the normal level [4,5]. Several of the biological properties ascribed to C-reactive protein resemble those of immunoglobu-

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lins. Like the immunoglobulins, C-reactive protein possesses the ability to promote reactions of precipitation [3] and agglutination [6], bacterial capsular swelling [7], phagocytosis [8] and complement fixation [9].

Structurally C-reactive protein appears to be an annular disc containing five identical subunits [5], the aminoacid sequence of which has been reported [10]. In its cyclic pentameric form C-reactive protein has a specific affinity for phosphorylcholine, which is calcium ion-dependent [9].

So far as we are aware no studies have been made of the thermal properties of human C-reactive protein. Three techniques have been used in this investigation, differential scanning calorimetry (DSC), fourier transform infrared spectroscopy (FTIR), and surface plasmon resonance spectrometry (SPR). DSC is the technique most commonly used to study the thermal properties of proteins [11]. The sample pan, containing hydrated protein, and the reference pan are heated at a fixed rate through the temperature at which the protein denatures. By monitoring the differential power input to the pans it is possible to determine the denaturation temperature of the protein and the enthalpy change upon denaturation. In the work reported here measurements on the protein in the ligand bound (plus phosphorylcholine and calcium) and ligand-free states have been used to show how the presence of the ligands affects the thermal stability of the protein.

Fourier transform infrared spectroscopy, aided by powerful data handling techniques, namely spectral deconvolution and differentiation, has become a widely used method for studying the secondary structure of proteins [12]. The amide I band (from 1700 to 1620 cm^{-1}) is due almost entirely to the C=O stretching vibration of the peptide linkages that form the protein backbone. This band is known to be sensitive to small variations in geometry and hydrogen bonding of carbonyl groups [12]. Here the amide I band has been used to study the effect that a linear increase in solution temperature has on the peptide backbone of the protein. The temperature range overlapped the calorimeter heating scans enabling a direct comparison of heat flow and spectral data to be made.

Surface plasmon resonance spectrometry uses an apparatus in which a beam of monochromatic light from a laser source is reflected from a metal surface. In the work to be described here the metal is silver. At one particular angle the parallel wave vector of the incident light is in resonance with surface plasmons and the light, which is normally reflected into a detector, is absorbed (plasmons are surface charge density waves which are a function of the metal and of the refractive index of any coating on the metal). The angle at which this occurs is termed the surface plasmon angle. Since the surface plasmon angle is dependent on the refractive index of any layer on the metal, changes in the nature of this layer, such as the binding of a ligand, cause a change in critical angle. For our work a phosphorylcholine derivative has been synthesised [13] with a thiol group. Reaction of the thiol group with silver places a covalently bound layer of phosphorylcholine on the metal surface. The interaction of C-reactive protein with this layer was studied by first setting the incident angle of the laser beam near the critical angle and then monitoring the changes in the intensity of the reflected light with time as the layer was brought into contact with a solution of the protein. The objective was to establish what effect, if any, heating had on the protein–ligand interaction.

2. Materials

2.1. C-reactive protein

Human C-reactive protein was purified from plasma derived from patients with an acute phase response undergoing plasmaphoresis. The plasma was first converted to serum by incubation at 37°C following addition of protamine sulphate (0.60 $\mu g\ cm^{-1}$) and calcium chloride (15 mM).

Using a modification of Volanakis et al. [14], serum was passed down a PC-Sepharose column (60 cm^3) washed with Tris buffered saline (TBS) containing 1 mM $CaCl_2$ and eluted with TBS containing 10 mM EDTA. The elute was diluted with a 50% volume of distilled water and added to a DEAE cellulose column equilibrated in 10 mM Tris–0.1 M NaCl. The protein was eluted with a linear gradient to 10 mM Tris–1M NaCl. Finally, the major peak was passed through an S-300 gel filtration column equilibrated in 10 mM Tris–0.15 M NaCl. The product thus obtained was free of phosphorylcholine.

2.2. Phosphorylcholine

In experiments with both the calorimeter and infrared spectrometer phosphorylcholine was used in its glycerol-bound form, glycero-phosphorylcholine (GPC). GPC is the polar head group of the most common membrane phospholipids, the diacyl phosphatidylcholines. GPC, free of contamination by other ions (such as calcium), was obtained as a dry-free running powder from Genzyme (UK) plc. TLC on silica gel plates using alcohol/water mixtures as eluent did not reveal any impurities.

3. Methods

3.1. Calorimetry

Human C-reactive protein was obtained as a solution (2.5 mg cm^{-1}) in TBS (10 mM Tris, 100 mM NaCl, pH 7.4 @ 20°C). For calorimetry it was concentrated in the following way: 0.5 cm^3 of protein solution was placed in each of two amicon microcon filter concentrators (10 kdalton cutoff) mounted in eppendorf tubes. They were spun down at 7000 g for 30 min at room temperature. The filtrate was discarded and the filters inverted into fresh eppendorf tubes and spun at 1000 g for 3 min to remove concentrated protein solution. The final volume of the solution was 50 μl, giving a solution concentration of 50 mg cm^{-1}. Several scans were also made at a concentration of 5 mg cm^{-1} (10-fold dilution) to establish if the thermal properties of the protein in this concentration range were concentration-dependent.

Samples for calorimetry (20 μl) were hermetically sealed inside stainless steel high pressure calorimetry pans. Heating and cooling scans were carried out on a Perkin-Elmer DSC7 scanning calorimeter interfaced to a Perkin-Elmer 7700 computer via a TAC7 instrument controller. Heat flow versus temperature data were digitised and stored on the computers hard disc. The onset temperature, the temperature at which the heat flow in the transition was at a maximum and the enthalpy changes of transitions were calculated using software supplied by Perkin-Elmer. The data was transferred to an 80 486 personal computer for differentiation and further analysis using Grams/386 software. The temperature scale was calibrated using indium as a standard and the enthalpy changes were quantified by comparison with indium ($\Delta H = 28.45$J g^{-1}). All samples were scanned at 5°C min^{-1}. Heating and cooling scans were made and at the conclusion of the first set of scans the samples were equilibrated at room temperature for several days after which a further heating scan was made. Single scans were also made at 2.5°C min^{-1} and 1°C min^{-1} to study the influence of scanning rate on the temperature at which the protein denatured.

3.2. Fourier transform infrared spectroscopy

Fourier transform infrared spectra were recorded on a Nicolet 740 FTIR spectrometer equipped with a TGS detector, controlled by a Nicolet 680 Spectral Workstation running Nicolet SX FTIR software. Water vapour contributions to the region of interest were minimised by continuously purging the spectrometer with medical grade air, dried using a Balston air dryer. A half hour purge of the instrument was carried out before scanning was started. Background and sample interferograms were recorded alternatively by utilising a sample shuttle (shuttling every 16 or 64 scans). The sample cell was a pair of calcium fluoride windows with a 6 μm deep depression ground into one window. Thermal control was achieved by surrounding the sample cell with a water jacket attached to a Haake CH1F3 circulating water bath. The water bath temperature was controlled by means of an IBM 8088 personal computer equipped with a digital to analog card, running in-house thermal control software. The samples used for the infrared measurements were identical in composition to those used for calorimetry. A 4 μl sample was pipetted into the 6 μm depression in one of the windows. The windows were then carefully brought together ensuring no air bubbles formed.

Thermal denaturation studies were carried out by collecting 1024 interferograms of the sample and background (shuttling every 16 scans) at 20°C, then heating the sample over a linear temperature gradient from 60°C to 90°C in a period of approximately 12 h. During the run interferograms were collected continuously (shuttling every 64 scans), and sets of 512 scans were co-added and fourier transformed to give 20 averaged spectra, each with a temperature range of approximately 1.5°C. Spectra were transferred to a personal computer for data conversion and analysis.

The appropriate buffer spectrum was digitally subtracted from the protein spectrum semi-automatically. In-house software, controlling Nicolet PCIR software was used to deconvolve the spectra.

3.3. Surface plasmon resonance spectroscopy

Surface plasmon resonance spectra were recorded using an Irlam Instruments single beam SPR spectrometer equipped with a 630 nm laser source, servo turntable and photoelectric detector, controlled by an Acorn Archimedes A410 personal computer running Irlam Instruments OXDAQ software. Angle scans were recorded between 65° and 73° with a resolution of 0.01°. Time scans were recorded between 0 and 480 s with a resolution of 0.5 s. Measurements were performed using 0.25 mg cm^{-1} solutions of C-reactive protein in Tris buffer (10 mM Tris, 5 mM $CaCl_2$, pH 7.4). The protein solution flow rate through the SPR cell was 0.24 cm^3/min. For each experiment four slides were analysed and the data averaged. The slides were obtained from Johnson and Johnson (Pollards Wood Laboratories, Nightingales Lane, Chalfont St. Giles, Buckinghamshire, UK) and consisted of 2.5 cm×2.5 cm glass squares coated on one side with 40 nm of silver. The slides were placed vertically in a PTFE well which was then filled with a 10 mM solution of thiol in methanol. After 4 h, they were removed, rinsed thoroughly with methanol and allowed to dry in an atmosphere of nitrogen. The slides were then index matched to the prism of the SPR instrument using ethyl salicylate and allowed to reach thermal equilibrium with the instrument over a period of 5 min. Experiments were then conducted.

4. Results

4.1. Calorimetry

Thermograms of human C-reactive protein in buffer and in buffer containing 5 mM molar calcium chloride, 5 mM GPC and both calcium salt and GPC are shown in Fig. 1. Clear denaturation endotherms were evident in all heating scans, the temperature of maximum heat flow increasing from 88.4°C in the absence of calcium ions and GPC to 90.25°C with calcium, 93.46°C with GPC and 95.14°C with both calcium and GPC. The presence of these two salts also increased the enthalpy change on denaturation. The denaturation enthalpy change of the protein was 17.62J g^{-1}, which was increased to 19.86, 18.39 and 25.93 J g^{-1} by the presence of, respectively, calcium ions, GPC and both GPC and calcium. Denaturation was always irreversible whether or not the salts were present, either on their own or in combination: i.e., there was no exotherm in the cooling curve and even after standing at room temperature for several days no endotherm in a second heating scan.

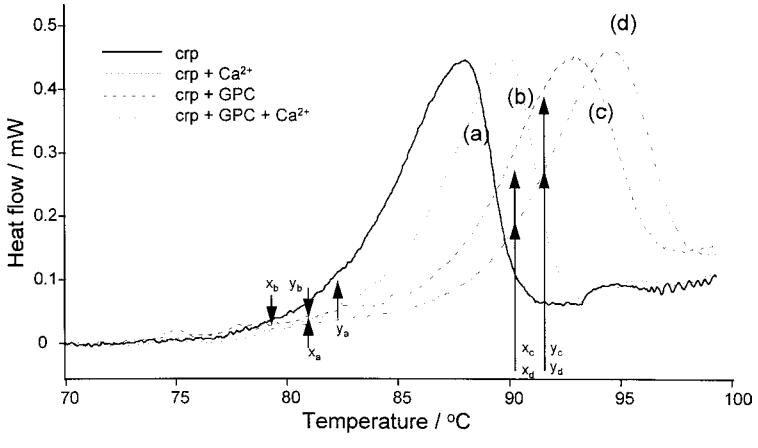

Fig. 1. Thermograms (heating) of human C-reactive protein in Tris buffer plus: (a) nil; (b) 5 mM $CaCl_2$; (c) 5 mM GPC; (d) 5 mM $CaCl_2$ and 5 mM GPC. *x* and *y* denote the temperature range over which the greatest change was detected in the infrared spectrum of the protein on heating.

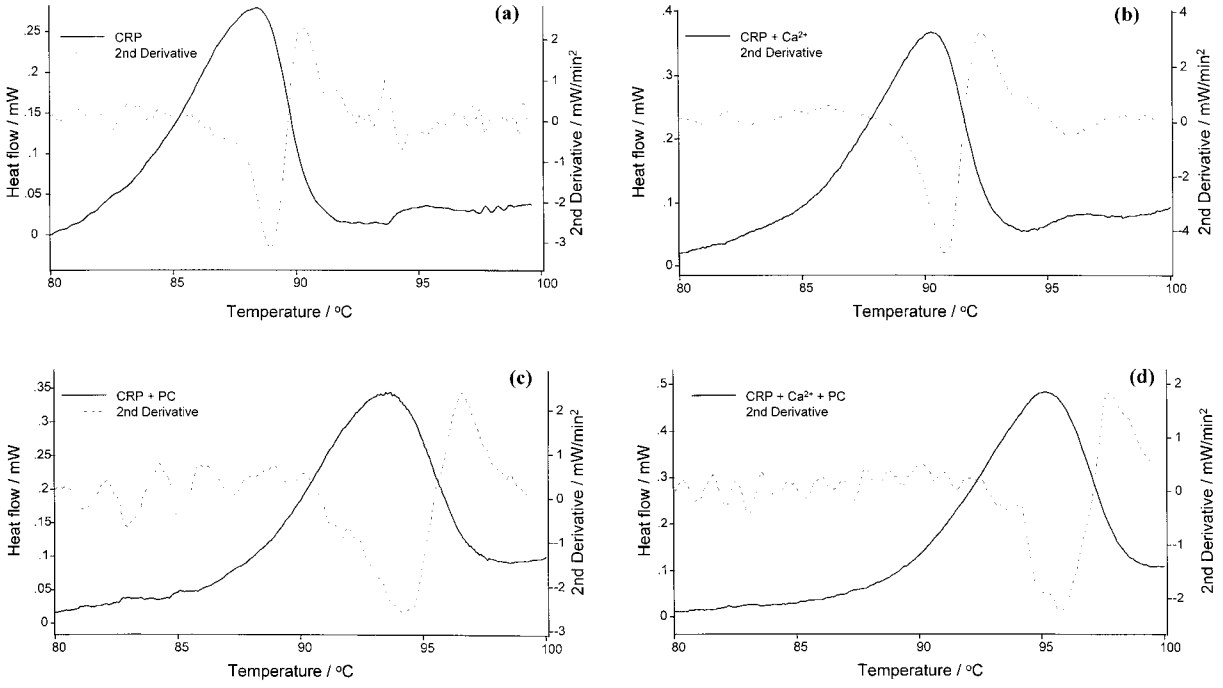

Fig. 2. Thermograms (heating) and thermogram second derivatives of human C-reactive protein in Tris buffer plus: (a) nil; (b) 5 mM $CaCl_2$; (c) 5 mM GPC; (d) 5 mM $CaCl_2$ and 5 mM GPC.

Addition of GPC made protein denaturation a less co-operative process, the transition occurring over a greater temperature range. The difference between the peak and the onset temperatures are: buffer only 4.98°C, Ca^{2+} 4.89°C, GPC 5.04°C and CA^{2+} + GPC 5.50°C. The broadening of the transition was associated with an increase in its complexity. Fig. 2 shows second derivatives of the endotherms displayed in Fig. 1. It is clear that for the protein in buffer only or in buffer with added calcium ions the second derivative has only one peak. When GPC is present, however, either with or without added calcium, the second derivative traces are more complex with at least one, possibly two additional peaks.

To establish whether the elevation of the denaturation temperature was due to GPC binding to the active site of C-reactive protein or was a common effect likely to be observed with any protein, heating scans were made on albumin both with and without added GPC. In Tris buffer, at the same protein and GPC concentrations employed with C-reactive protein, no difference in either the onset temperature or the temperature of maximum heat flow of denaturation was detected when GPC was added to the protein solution.

Thermograms obtained at 10-fold dilution of the protein had a lower signal to noise ratio. However, denaturation endotherms could still be clearly seen and it was found that at this lower concentration denaturation occurred at lower temperatures. The denaturation temperatures were: buffer only 85.0°C, $+Ca^{2+}$ 87.25°C, +GPC 91.5°C and +GPC and Ca^{2+} 92.2°C. Relative to the corresponding values at 50 mg cm^{-1} these values represented decreases of 3.4°C, 3.0°C, 1.99°C and 2.94°C.

4.2. Fourier transform infrared spectroscopy

Fig. 3 shows the effect of temperature on the infrared spectra of C-reactive protein in the region 1670–1610 cm^{-1}. As well as the buffer spectrum (a), spectra in buffer solution containing calcium chloride, GPC and both calcium salt and GPC are also shown in (b), (c) and (d), respectively. At temperatures below the denaturation temperature addition of the salts caused a small decrease in the absorbance of the peak at

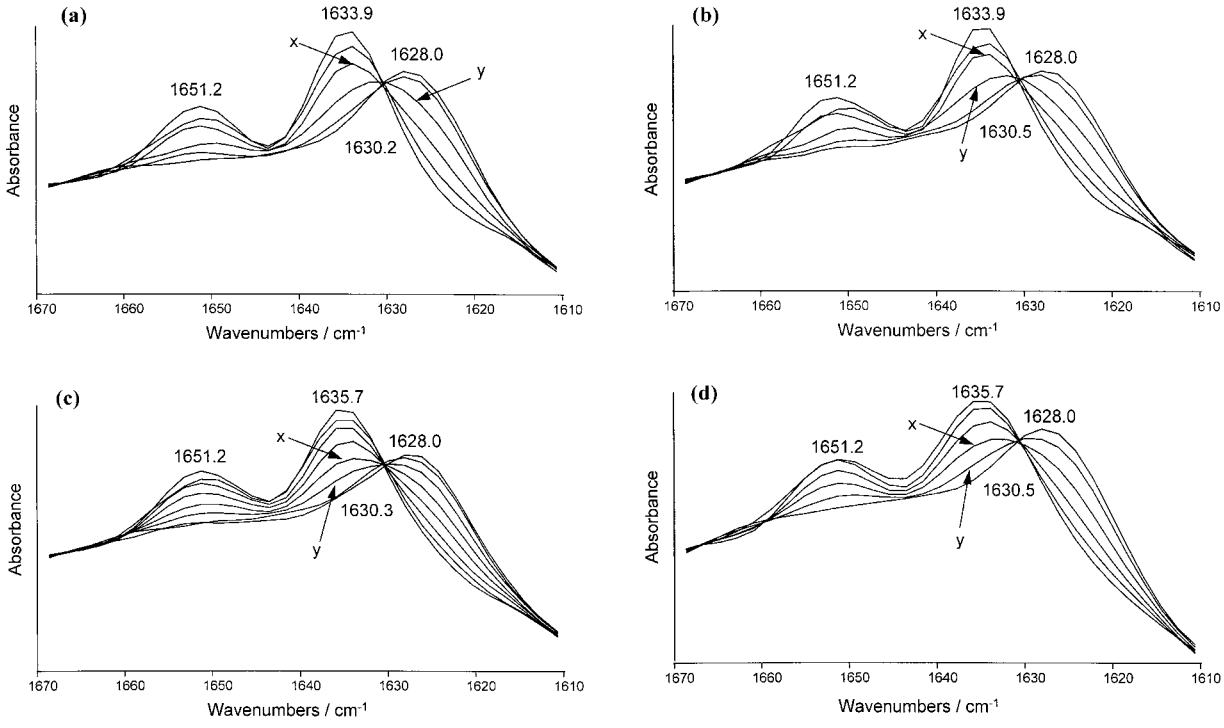

Fig. 3. Fourier transform infrared spectra of C-reactive protein in Tris buffer plus: (a) nil, at temperatures from the top down of 71.25°C, 72.25°C, 75.75°C, 77.25°C, 78.75°C and 84.75°C; (b) 5 mM $CaCl_2$, at temperatures from the top down of 72.75°C, 74.25°C, 75.75°C, 77.25°C, 78.25°C and 79.75°C; (c) 5 mM GPC, at temperatures from the top down of 72.75°C, 75.75°C, 78.75°C, 81.75°C, 83.25°C, 84.75°C, 86.25°C, and 87.75°C; (d) 5 mM $CaCl_2$ and 5mM GPC, at temperatures from the top down of 81.75°C, 83.25°C, 84.75°, 86.25°C, 87.75°C and 89.25°C.

1633.9 cm^{-1} and a corresponding increase in the absorption at 1651.2 cm^{-1}. The magnitude of these changes followed the order Ca^{2+}+GPC > GPC > Ca^{2+}. The presence of GPC caused the absorption at 1633.9 to shift to 1635.7 cm^{-1}.

Fig. 3(a)–(d) are qualitatively similar. As the protein was heated a new band appeared at 1628 cm^{-1}. It steadily increased in intensity until it reached a maximum where further heating caused no more change in its strength. The increase in this band was accompanied by a corresponding decrease in the intensity of the 1651 and 1633.9/1635.7 cm^{-1} bands. By the time the 1628 cm^{-1} band had reached its maximum strength the 1651 and 1633.9/1635.7 cm^{-1} bands had disappeared. There was an isosbestic point at 1630.2 cm^{-1}. The temperature intervals over which the largest shift occurred in the maximum absorption of the spectrum are marked *x–y* in each of the four sets of spectra. These temperature intervals, after correction, are also plotted on the corresponding thermograms in Fig. 1. A correction had to be made as the calorimetric and spectral data were obtained at different scanning speeds. Thermograms were recorded at 5°C min^{-1}, while collection of the full infrared data set took nearly 12 h, effectively zero scanning speed. By carrying out DSC scans at 2.5°C min^{-1} and 1.0°C min^{-1} in addition to 5°C min^{-1}, it was established that there was a linear relationship between scanning speed and the temperature of maximum heat flow during denaturation. Extrapolating to zero scanning speed gave the amount by which the *x–y* intervals had to be shifted along the temperature axis (+5.42°C) so that comparisons could be made with thermograms.

4.3. Surface plasmon resonance spectroscopy

Fig. 4 shows SPR spectra for adsorption of C-reactive protein onto slides coated with pure phosphorylcholine dodecanethiol. There was significant adsorption of the native protein onto this surface

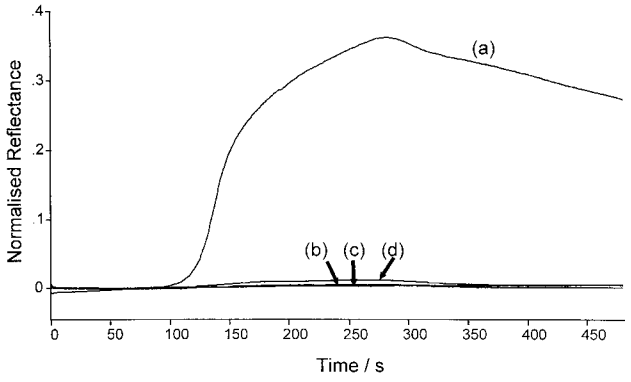

Fig. 4. Adsorption onto 100% phosphorylcholine dodecanethiol-coated silvered microscope slides of C-reactive protein in Tris containing $CaCl_2$ (concentration 5 mM): (a) native protein, (b) native protein + GPC, (c) denatured protein, (d) denatured protein + GPC. GPC concentration 5 mM.

(a). However, when the protein was denatured or complexed with GPC this adsorption was virtually eliminated. Fig. 5 presents a similar set of data to Fig. 4 except in this instance the phosphorylcholine dodecanethiol had been diluted with 10% dodecanethiol before the slides were coated. Diluting the coating in this way caused a reduction in the amount of native protein adsorbed but lead to significant adsorption of some denatured protein and protein complexed with GPC. Washing the coating with buffer alone (region after approximately 250 s) removed bound native protein but not denatured or complexed protein. For the puposes of comparison we have also looked at the interaction of albumin with these surfaces, Fig. 6. The presence of GPC had no bearing on the adsorption characteristics of albumin. There was no adsorption onto the surface coated with pure phosphorylcholine dodecanethiol but adsorption did occur on the coating diluted with dodecanethiol. The amount of albumin adsorbed was similar to the amount of denatured and complexed C-reactive protein which adsorbed on this surface and, like these C-reactive protein deposits, washing with buffer did not remove it.

5. Discussion

5.1. Differential scanning calorimetry

Thermograms obtained by DSC show clearly that the denaturation temperature of C-reactive protein was increased substantially by addition of GPC and cal-

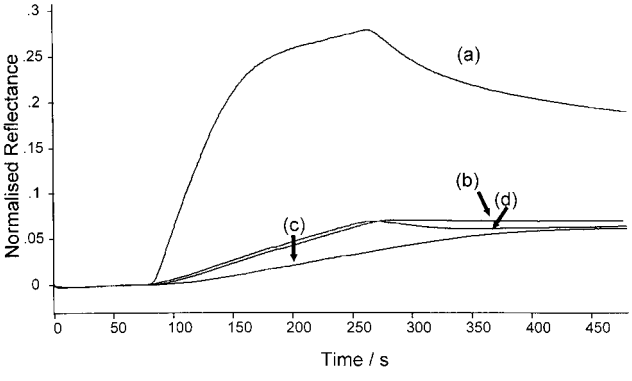

Fig. 5. Adsorption onto 90% phosphorylcholine dodecanethiol: 10% dodecanethiol-coated silvered microscope slides of C-reactive protein in Tris containing $CaCl_2$ (concentration 5 mM): (a) native protein, (b) native protein + GPC, (c) denatured protein and (d) denatured protein + GPC. GPC concentration 5 mM.

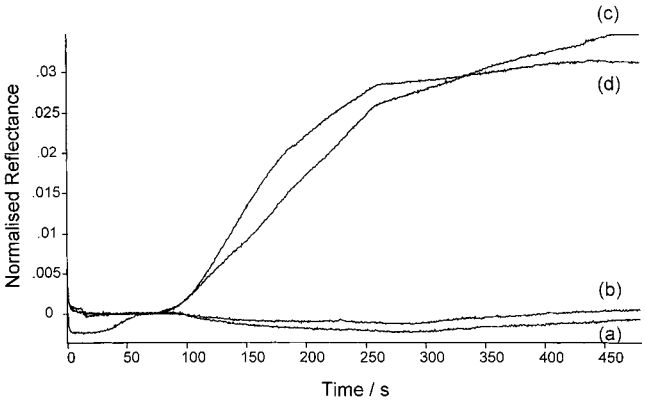

Fig. 6. Adsorption onto silvered microscope slides of albumin in Tris containing $CaCl_2$ (concentration 5 mM): (a) 100% phosphorylcholine dodecanethiol coated slides, (b) 100% phosphorylcholine dodecanethiol-coated slides, GPC added to buffer, (c) 90% phosphorylcholine dodecanethiol: 10% dodecanethiol-coated slides, (d) 90% phosphorylcholine dodecanethiol: 10% dodecanethiol-coated slides, GPC added to buffer. GPC concentration 5 mM.

cium salt to the buffer. Calorimetry, therefore, demonstrates binding of phosphorylcholine to the protein and shows that this binding results in an increase in thermal stability. However, addition of GPC without calcium salt also caused an, albeit smaller, increase in denaturation temperature. It is clear then that either EDTA used in the purification of the protein did not entirely strip it of calcium ions or that the protein subsequently picked up calcium from one of the buffer solutions used. It has long been known that for C-reactive protein to bind to its principal ligand, the phosphorylcholine residue of either lipids, proteins or polysaccharides, calcium ions are required [9]. However, calcium salt alone added to the buffer also increased the denaturation temperature of the protein, so binding sites on the protein for calcium were not saturated by the traces of calcium left after purification.

The entropy change (enthalpy change divided by denaturation temperature) on denaturation is increased by 40% when calcium and GPC (both 5 mM) are added to the buffer. This implies a significantly greater disordering upon denaturation. In principle this could be due either to the salts causing an ordering of the protein in its native form or a disordering of the denatured form. The last proposition seems unlikely, suggesting that binding of calcium and GPC to the protein causes a structural change which leads to an increase in order in the polypeptide chain or its substituents.

The binding of phosphorylcholine to the protein can also be demonstrated by differentiating the denaturation thermograms. In the absence of GPC, either with or without calcium, the denaturation process is highly co-operative and only one peak is evident in the second derivative. The presence of GPC, again either with or without calcium, decreases the co-operativity of the transition. It is spread over a greater temperature range and apparently now occurs in more than one stage, there being multiple peaks in the second derivative of the thermogram. This suggests that binding of GPC causes the formation of non-interacting domains within the structure of the protein.

Human C-reactive protein, especially in the presence of calcium and GPC, is a particularly stable protein, i.e., intramolecular binding forces are strong. Its denaturation temperature is 32°C higher than that of albumin when measured under similar conditions.

The reduction in the denaturation temperature of 3.4°C seen when the protein concentration was diluted 10-fold shows that in the concentration range studied here the denaturation temperature of the protein is concentration-dependent. This is most likely the result of aggregation. Given the sensitivity of the calorimeter used for this study it is impossible to work at much lower protein concentrations and obtain noise-free thermograms. To obtain unaggregated protein, dilutions of 100-fold or more might be required. In the FTIR work to be discussed next lower protein concentrations could only have been reached if deuterium

oxide had been used in place of water. However, there is a possibility that the degree of hydrogen, deuterium exchange would increase as the protein denatured making interpretation of spectra impossible in this solvent. The increases in denaturation temperature caused by the addition of the proteins ligands were, within experimental error, unaffected by the dilution. Therefore for the purposes of this work the absolute protein concentration is not significant.

5.2. Fourier transform infrared spectroscopy

The amide I region of the spectrum contained two major peaks. The peak at 1633.9 cm^{-1} is thought to arise from the polypeptide chain arranged in a β-sheet conformation and the 1651.2 cm^{-1} absorption from an α-helix [12]. Addition of GPC had no effect on the 1651.2 cm^{-1} absorption but did shift the absorption attributed to β-sheet by 1.8 cm^{-1} to shorter wavelength. It seems therefore that the site where GPC binds is in a region of the protein where the packing is β-sheet. This is in accord with a recent article [15] describing X-ray diffraction measurements made on crystals of this protein. Some of the peptide residues which are identified as part of the pc binding site are also amongst those which form the two sheets of antiparallel β-strands in the core of the protein. Calcium ions either with or without GPC had no detectable effect of the absorption maximum of either band.

We believe that the marked change that occurs in the spectrum of the protein as it is heated is a result of its denaturation. There is good agreement between the temperatures at which the spectral changes and denaturation endotherms occur. The presence of an isosbestic point in the spectra demonstrates that upon denaturation α-helical and β-sheet structures are converted directly without any intermediate into the form which absorbs at 1628 cm^{-1}. The fact that α-helix and β-sheet simultaneously convert to this form and that the enthalpy change on conversion is large, ∼20 J g^{-1}, indicate that the transformation that gives rise to the 1628 cm^{-1} form is of the nature of a change in phase and not a minor structural change. The entropy of the denatured state is much larger (∼70 mJ $g^{-1}K^{-1}$) than that of the native protein. Therefore, the amide groups which give rise to the absorption at 1628 cm^{-1} are in a structure with a great deal more freedom of movement than the α-helix or β-sheet found in the native form of the protein. This implies a marked reduction in the amount of amide–amide hydrogen bonding and so we believe that the 1628 cm^{-1} absorption arises from completely disordered polypeptide chain where practically all hydrogen bonds to the amide groups are with water.

The infrared spectra confirm the conclusion reached by DSC, that the presence of GPC increases the denaturation temperature of the protein. However, unlike calorimetry, infrared spectroscopy did not show any increase in the denaturation temperature when calcium ions were added to the buffer. This is consistent with the observation that only GPC, and not calcium, is capable of altering the absorption maximum of one of the spectral bands. This suggests that while calcium binds to the protein, unlike GPC, it does so in a manner which does not influence the hydrogen bond network of the amide residues. It also should be noted that while calcium is capable of increasing the denaturation temperature measured by calorimetry it does not lead to formation of domains which denature at slightly different temperatures as GPC does, as the presence of calcium did not affect the shape of the second derivative of the denaturation endotherm.

Comparison of the calorimetric and DSC data shows that significant rearrangement of the amide bonding network of the protein occurs before the major heat flow of denaturation. Without GPC the *x–y* intervals (points of maximum change in the infrared spectrum, compare Figs. 1 and 3) lie at approximately the onset temperature of the denaturation. Adding GPC shifts the *x–y* intervals further along the calorimeter trace implying that the presence of GPC strengthens hydrogen bonding interactions between amide groups.

Dong et al. [16], have recorded the spectrum of human C-reactive protein before and after addition of phosphorylcholine and calcium. Unlike us they find that the presence of calcium causes a blue shift of 1 cm^{-1} in the position of the β-sheet band. They do not report the 2 cm^{-1} blue shift in this band we found upon binding of GPC. However, their experiments were carried out in deuterium oxide rather than water. It is possible that in D_2O the dissociation constants for the ionic equilibria involved are different or that binding of the ligands to the protein in D_2O solutions cause an increase in the extent of hydrogen–deuterium exchange.

5.3. Surface plasmon resonance spectroscopy

The traces in Fig. 4 show clearly that C-reactive protein adsorbs specifically to phosphorylcholine coatings on silvered microscope slides. Adsorption only occurred when the protein was in its native conformation and its active site was unoccupied. It was possible to force non-specific adsorption by reducing the concentration of phosphorylcholine on the surface. That non-specific adsorption occurs under these conditions is demonstrated by the similarity of the adsorption isotherms of denatured and complexed C-reactive protein and the adsorption isotherms of albumin. Adsorption of these proteins could also be shown to be different in character to the adsorption of native C-reactive protein by washing the coatings with buffer. Adsorbed layers of native C-reactive protein were removed by washing but coatings of albumin and denatured or complexed C-reactive protein were stable. One other indication that the adsorption of native C-reactive protein was via phosphorylcholine was the reduction in the amount adsorbed when the concentration of phosphorylcholine in the surface was reduced (compare Fig. 4(a) and Fig. 5(a)).

The failure of the protein to bind specifically to phosphorylcholine after heating demonstrates that the packing of peptide residues at the ligand binding site was irreversibly disrupted by denaturation. This supports our view that in the high temperature form of the protein that absorbs at 1628 cm^{-1} there is little of the order left that exists in the native structure that absorbs at higher wave numbers.

6. Conclusion

The work described here shows that, as with many of the other properties of this protein studied previously, measurements of its thermal properties also have a role to play in understanding its structure and function. Calorimetry yields valuable quantitative information about the forces holding this protein together and how these forces change when the ligands calcium and phosphorylcholine are bound. Both ligands act to enhance intramolecular binding forces. The infrared measurements, like the calorimetry, demonstrate that GPC binds to C-reactive protein and in so doing reinforces the structure of the protein. However, unlike calorimetry infrared spectroscopy did not reveal any interaction between calcium ions and C-reactive protein. It has to be concluded, therefore, that calcium binding occurs without any change in hydrogen bonding in the polypeptide backbone of the protein. The SPR technique was able to detect the interaction between C-reactive protein and a surface containing phosphorylcholine groups. The structural changes that take place when the protein is denatured are sufficient to destroy its interaction with phosphorylcholine.

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