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Recombinant factor VIII SQ — the influence of formulation parameters on structure and surface adsorption

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Abstract

The main aim of this paper was to investigate the influence of temperature, pH and ionic interactions on the structural stability and surface adsorption of a recombinant factor VIII product, r-VIII SQ. The interaction of r-VIII SQ with glass and air interfaces, and possible means of increasing the stability of the formulation, were also investigated. The stability of r-VIII SQ was followed by measuring the biological activity (VIII:C), by circular dichroism (CD) studies and by the measurement of surface tension using the pendant drop method. The results show that the surface tension decreased exponentially with time; this decrease was more pronounced above 20°C, indicating increased conformational flexibility of the protein with increased temperatures. Far UV CD spectra were not influenced in the range 5–55°C and near UV CD measurements did not indicate structural changes below 45°C. During agitation at 25°C, VIII:C was lost rapidly in formulations without a macromolecular additive. Nonionic surfactants such as polysorbate 80 and polysorbate 20 protected VIII:C to an equally high degree against surface adsorption. Albumin was less effective, but it is possible that this is because it is a protein itself and may have been affected by the agitation. The addition of 300 mg/ml of sucrose improved the long term stability of VIII:C, a finding most likely explained by the theory of preferential hydration. Near UV CD spectra at acidic or basic pH mainly indicated changes around 242 nm, especially at low ionic strength. Addition of 10 mM EDTA at pH 7 resulted in similar changes. This effect was completely reversed by the addition of an excess of Ca^{2+}, Sr^{2+} or Mg^{2+} ions. In conclusion, CD spectra and surface tension measurements of r-VIII SQ did not reveal any temperature-induced conformational changes in the temperature range 5–20°C; changes were first noted at elevated temperatures. Surface adsorption of r-VIII SQ during agitation was prevented by the addition of a nonionic surfactant. Preferential hydration improved the storage stability of the protein but did not directly prevent its surface adsorption. The structural integrity of the molecule was preserved at pH 7, at an increased ionic strength and in the presence of some divalent metal ions (Ca^{2+}, Sr^{2+} or Mg^{2+}).

Keywords: Protein stability; Factor VIII; Conformation; CD; Surface tension; Metal ions

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1. Introduction

Functional deficiencies in the factor VIII protein cause haemophilia A, a hereditary X-chromosome-linked bleeding disorder. During blood coagulation, factor VIII participates as an essential nonenzymatic cofactor in the enzyme complex that activates factor X. Therapeutic factor VIII concentrates contain several heterodimeric forms of the active factor VIII protein, each composed of a light chain of 80 kDa and a heavy chain of 90–200 kDa (Andersson et al., 1986). The heterogeneity of the latter chain is caused by limited proteolysis from the C-terminal end of the B-domain. Recombinant factor VIII SQ (r-VIII SQ) is a new genetically engineered factor VIII product that lacks the B-domain (Lind et al., 1995). This factor VIII derivative corresponds to the smallest active form, a metal ion-linked 80 and 90 kDa heterodimer, present in all factor VIII products for therapeutic use.

One obstacle in developing stable pharmaceutical formulations containing factor VIII is the large size and complexity of the molecule (Andersson et al., 1986). The biological activity of factor VIII is reliant on the presence of several different structural domains, and the molecule is sensitive to both chemical and physical degradation. The r-VIII SQ molecule consists of two glycosylated protein chains (containing a total of 1438 amino acid residues), seven disulphide bridges, five sulphydryl groups and a metal ion bridge. There is still some uncertainty regarding the identity of the metal ion linking the two protein chains (Bihoreau et al., 1994; Sudhakar and Fay, 1998). A further obstacle is the low concentration of factor VIII usually used, because significant losses may occur as a result of surface adsorption and/or denaturation (an average dose of 1000 IU r-VIII SQ corresponds to 0.02 mg/ml or 0.1 nmol/ml in solution; the specific activity corresponds to 15 000 IU/mg). Usually, at such low concentrations of protein, albumin is added to prevent surface adsorption and to increase the stability of the formulation. However, when albumin is added, several additional factors should be considered: (i) there is a risk of unknown infectious agents being included with the albumin; (ii) albumin is expensive; and (iii) the presence of albumin will restrict the characterisation of the finished product as there will be much more albumin than drug present. A replacement for albumin is thus required, as approximately 50% r-VIII SQ may otherwise be lost during sterile filtering (Österberg et al., 1997).

Earlier work has shown that the stability of r-VIII SQ can be improved by increasing the concentration of some divalent metal ions (Ca^{2+} and Sr^{2+}), increasing the ionic strength, and maintaining the pH at around 7 (Fatouros et al., 1997a). Stability studies showed that the decline in activity followed pseudo-first-order kinetics, but that the Arrhenius plot was nonlinear in the temperature range 5–20°C (Fatouros et al., 1997b). The cause of this may have been temperature-induced changes in the reaction mechanism or the rate-determining step, or there may have been alterations in the higher order structure. A correlation was found between the decline in the biological activity of r-VIII SQ (VIII:C) and the dissociation of the light (80 kDa) and heavy (90 kDa) chains of the molecule, especially at 5°C and outside the pH range of 6.5–7 (Fatouros et al., 1997a,b). This may indicate that alterations in the higher order structure of r-VIII SQ induced by low temperatures could lead to chain separation, resulting in the curved Arrhenius plot. Differential scanning calorimetry (DSC), however, did not indicate any transitions in the low temperature range. A shallow endotherm was first seen at approximately 56°C; this gradually transformed into a larger exotherm caused by irreversible aggregation and precipitation (maximum exotherm heat flow at 64.2°C).

In this study the structural stability of r-VIII SQ as a function of temperature, pH and ionic interactions was investigated using surface tension measurements and ultraviolet (UV) circular dichroism (CD) studies. The interaction of r-VIII SQ with glass and air interfaces and the means of increasing the stability of the formulation were also investigated.

2. Materials and methods

2.1. Materials

r-VIII SQ was produced by Pharmacia and Upjohn AB (Sweden) as described below. Sodium chloride, calcium chloride dihydrate, polysorbate 80, L-histidine, hydrochloric acid, sodium hydroxide and sucrose were of pharmacopoeial quality. Genapol 80, pluronic F 68, polysorbate 20, tris-(hydroxymethyl)aminomethane (tris), and ethylenediamine-tetraacetic acid tetrasodium salt (EDTA) were of analytical quality. Divalent metal ions of analytical grade were in the form of chloride salts. Water for injection or equivalent was used for all preparations.

Haemaccel® (Hoechst, Sweden), Haes®-steril (Meda, Sweden), and Human Serum Albumin (Pharmacia and Upjohn AB, Sweden) are clinically used as plasma volume expanders and were used as received. Haemaccel® contains polypeptides (mean molecular mass about 30 kDa) derived from bovine gelatin by thermal hydrolysis and cross-linked with hexamethylene diisocyanate. Haes®-steril contains poly (*O*-2 hydroxyethyl) starch (mean molecular mass about 20 kDa). Promiten® (Pharmacia and Upjohn AB) was used as received. It is a low-molecular-weight dextran (Dextran 1) used as a prophylactic agent against dextran-induced anaphylactic reactions.

The sterile filter used was 0.22 μm, Millex-GV (Millipore, Sweden). The container was an injection vial, glass type 1 (Ph. Eur. second edition and USP), stoppered with a bromobutyl rubber stopper (Helvoet FM 157, Belgium) and sealed with an aluminium flip-off seal. Nitrogen was of quality 5.5 (AGA, Sweden, less than 0.5 ppm of impurities). Buffer exchange was performed on a NAP™-5 Column (Pharmacia Biotech, Sweden).

2.2. Methods

r-VIII SQ was produced in Chinese hamster ovary (CHO) cells, cultivated in a serum-free medium at finite passage level. Purification was accomplished by several chromatographic steps using ion exchange, immunoaffinity, hydrophobic interaction and molecular size exclusion as separation principles. r-VIII SQ is highly purified with a specific activity of about 15 000 IU VIII:C/mg of protein. Bulk material of r-VIII SQ, with a factor VIII activity of 2500–10 000 IU/ml, was obtained from the final purification step and used either directly or stored frozen at −70°C. The elution buffer contained 18 mg/ml sodium chloride, 0.5 mg/ml calcium chloride dihydrate, 3 mg/ml L-histidine and 0.2 mg/ml polysorbate 80. Polysorbate 80 was omitted for selected experiments.

Formulations containing the various excipients and the desired factor VIII activity were manufactured by mixing the drug product with the appropriate buffers. Solid sodium chloride or carbohydrate was added directly to the solution for some formulations. The pH was adjusted with hydrochloric acid (1 M) or sodium hydroxide (1 M). The solutions were sterile filtered, and 2 ml was dispensed in 10 ml molded glass vials. The samples were assayed immediately after preparation and after agitation at 25°C (controlled incubator). Agitation was provided by placing the vials on a vibrating plate (Immuno vib 4, Labassco) set at 600 rpm, which created a swirling vortex in the solution. Reference samples stored without agitation were also assayed. Previous studies have shown that r-VIII SQ is susceptible to oxidation (Fatouros et al., 1997a). The stability of VIII:C during agitation was therefore first investigated in glass vials with a headspace containing either nitrogen or air. These conditions had no significant influence on the stability of VIII:C over the 144 h (6 days) of agitation at 25°C (data not shown). In subsequent studies, air was used in the headspace, thus simplifying sample withdrawal. For longer storage-stability studies, the oxygen content was reduced by reducing the pressure (0.02 bar) and introducing nitrogen over several cycles before the vials were closed.

2.3. Analytical methods

Factor VIII activity (VIII:C) was assayed using a chromogenic substrate method (Coatest® Factor VIII, Chromogenix AB, Sweden). All samples were prediluted in factor VIII-deficient plasma. The VIII:C was expressed in international units

(IU) as defined by the international concentrate standard. The relative S.D. of the assay was about 7% for formulation samples. The recovery of VIII:C after storage was expressed as % recovery of the initial VIII:C value.

Surface tension was measured using the pendant drop method, performed at the Institute for Surface Chemistry (Sweden). Due to method requirements, a more concentrated preparation of r-VIII SQ (9500 IU/ml ($\approx$ 0.6 mg/ml)) was used in a formulation containing 18 mg/ml sodium chloride, 0.5 mg/ml calcium chloride dihydrate and 3 mg/ml L-histidine. The corresponding buffer containing the same formulation but excluding the protein was also investigated. Samples were first equilibrated at the selected temperature for 15 min. Subsequently, a sessile drop was formed from a micrometer syringe in front of a light source (He–Ne laser) and the process was recorded with a video camera. The shape of the drop was determined and the surface tension was calculated by centering the apex of the drop to a coordinate system and using a modified Young–Laplace equation. Measurements were performed for 15 min in duplicate with the exception of one sample which was followed for 1 h.

CD spectroscopy was performed on a JASCO J-720 (Japan). In order to obtain a spectrum for r-VIII SQ, a more concentrated preparation (9500 IU/ml ($\approx$ 0.6 mg/ml)) was used in a formulation containing 18 mg/ml sodium chloride, 0.5 mg/ml calcium chloride dihydrate and 3 mg/ml L-histidine. The temperature was controlled by a Neslab RTE-110 water bath (Chemical Instruments, Sweden) monitored by a temperature probe at the cuvette holder. A quartz cuvette with path length of 10 mm was used (UVIR, Sweden). Scans were obtained with a 1 nm slit and 50 mdeg sensitivity. Five scans were obtained for each formulation and correction for background dichroism was made by subtracting the CD spectrum recorded for the corresponding buffer solution. Background noise was reduced in all CD spectra by use of the Fourier transformation algorithm in the instrument's J-700 software. Owing to spectral interference, the L-histidine buffer was replaced by 10 mM Tris for far UV CD and for some of the near UV CD studies. Buffer exchange was performed on a NAP™-5 Column as specified by the manufacturer.

3. Results and discussion

3.1. Stability of VIII:C during agitation

Proteins are known to adsorb to a variety of surfaces, both hydrophilic and hydrophobic, an effect which has more impact when the protein concentration is low (Horbett, 1992). The surfaces of most interest in pharmaceutical development programs are those encountered during sterile manufacturing and in the final container, assuming that purification is adequately undertaken. One of the main obstacles is the sterile filtering step, where the protein encounters a very large surface area. Adsorption can also occur at the interface with air, which may be regarded as a hydrophobic surface, and this is often followed by denaturation and aggregation of the protein; this leads to changes in the surface tension which can be measured (Bagnall, 1977; Levine et al., 1991; Horbett, 1992). Increasing the surface area by agitation/shaking accelerates the adsorption process (Henson et al., 1970). For parenteral products such as factor VIII the final container is usually made of glass with a rubber closure. Glass presents a hydrophilic surface which may be modified if required by the addition of a surface coating such as silicone.

Many macromolecules are known to stabilise therapeutic proteins against surface denaturation, and some considered to have a low potential for adverse effects were investigated here, i.e. they should be approved for parenteral use and not stimulate an immune response. An albumin formulation was included for reference since albumin is widely used as a protein stabilising agent and, furthermore, it is known to stabilise r-VIII SQ against surface adsorption during sterile filtering (Österberg et al., 1997). VIII:C was lost rapidly during agitation at 25°C in the formulations without a macromolecular additive, and also in formulations containing Haes®-steril and Promiten® (Fig. 1A). The inefficiency of these additives to stabilise r-VIII SQ during agitation could be ex-

plained by their hydrophilicity and lack of surface activity. Both Haes®-steril and Promiten® are macromolecules derived from carbohydrates and their hydrophilic nature probably lessens their driving force towards the hydrophobic air interface and they are thereby not preventing the surface adsorption of the protein. A decline in activity, although slower, also occurred in the corresponding non-agitated references (data not shown).

Nonionic surfactants, polysorbate 80 and polysorbate 20 (and also Pluronic F 68 and Genapol 80, data not shown), protected VIII:C to an equally high degree (Fig. 1A), probably against both glass and air interfaces (Thurow and Geisen, 1984). Albumin was less effective, but this may be explained by the fact that it is a protein itself and may have been affected by the agitation (Bagnall, 1977). The albumin molecule could be depleted from the solution by adsorption at the continu-

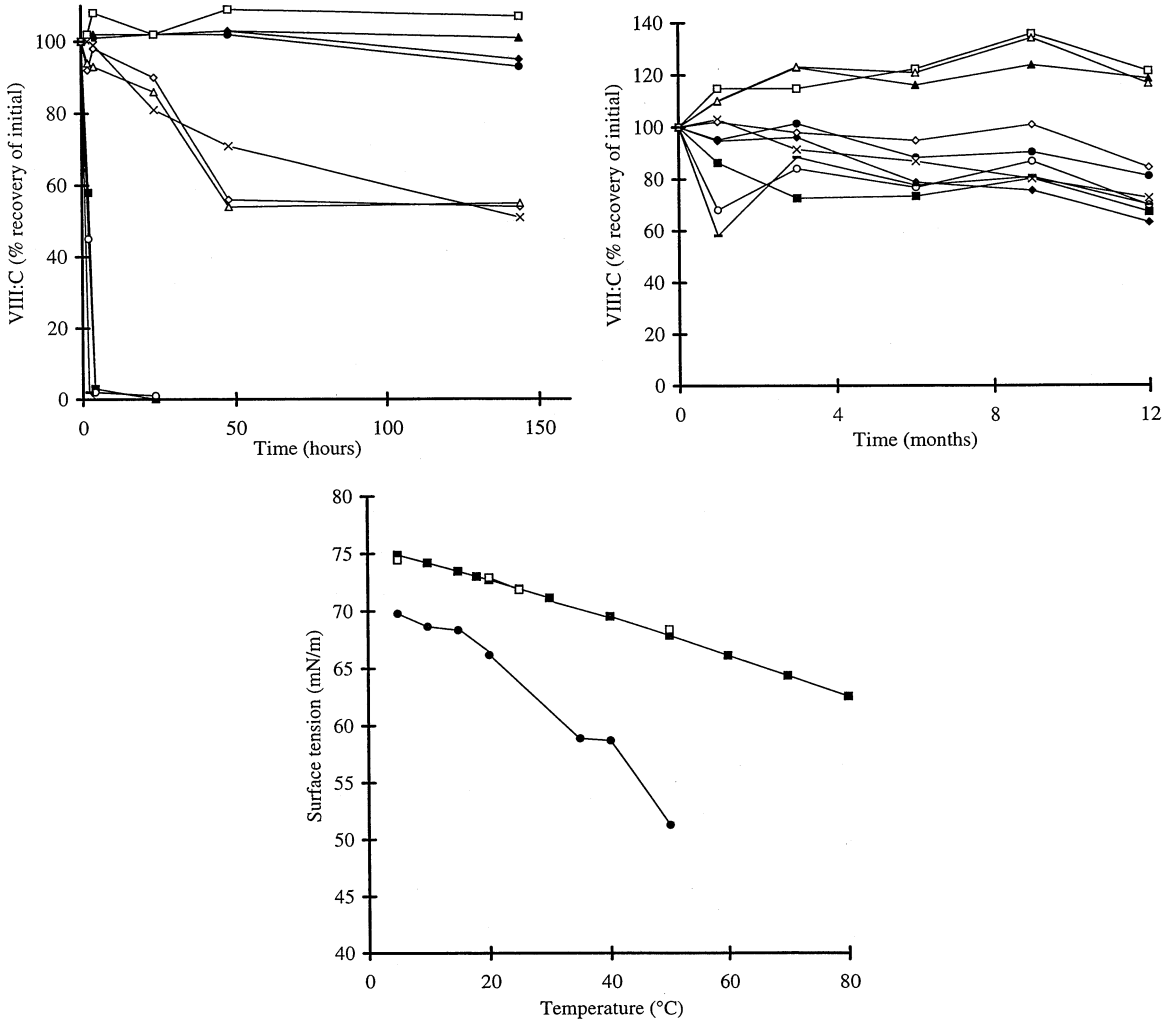

Fig. 1. Recovery of VIII:C in formulations containing macromolecular additives: (A) after agitation at 25°C for 144 h; (B) after storage at 5°C for 12 months. ■, no additive; ◆, 0.2 mg/ml polysorbate 80; ▲, 0.2 mg/ml polysorbate 80 with 300 mg/ml sucrose, ●, 0.2 mg/ml polysorbate 20; X, 20 mg/ml albumin; □, 20 mg/ml albumin with 300 mg/ml sucrose; ◇, 20 mg/ml Haemaccel®; Δ, 20 mg/ml Haemaccel® with 300 mg/ml sucrose; o, 20 mg/ml Haes®; and –, 20 mg/ml Promiten®. (C) Influence of temperature on the surface tension of water (■) (adapted from Handbook of Chemistry and Physics), formulation buffer (□), and r-VIII SQ (●).

ously formed new air interface and subsequent denaturation and precipitation, thereby losing its protective effect. It is interesting to note that albumin in combination with sucrose increased the stability of VIII:C during agitation. The formulation containing Haemmacel® showed a similar degree of protection on VIII:C as albumin but, in contrast, did not show a further increase in the recovery of VIII:C when sucrose was added. This difference could be explained by the fact that Haemaccel® probably lacks an ordered conformation which could be stabilised by sucrose (see below). Haemaccel® contains polypeptides (mean molecular mass about 30 kDa) derived from bovine gelatin by thermal hydrolysis and cross-linked with hexamethylene diisocyanate. The formulations containing Haes®-steril, Promiten® or albumin but without sucrose all developed large white particles after 48 h of agitation. This indicates that these macromolecules were themselves unstable during agitation, since the formulations which did not contain additives and the nonagitated references did not develop these particles.

The stabilising effect of albumin plus sucrose during agitation may have been a direct effect of preferential hydration on r-VIII SQ. Alternatively, it may have been caused by an indirect effect related to the stability of albumin. Preferential hydration is caused by some co-solutes, e.g. certain salts, polyalcohols, sugars and amino acids, which interact strongly with water and therefore tend to avoid contact with the protein (Arakawa and Timasheff, 1982). Minimising the protein surface presented to the solution reduces this thermodynamically unfavourable state and, coincidentally, stabilises the native protein conformation. This is illustrated by storage stability data obtained at 5°C for 12 months, in which the recovery of VIII:C was higher for the formulations containing 300 mg/ml sucrose (Fig. 1B). Although the stabilising effect provided by preferential hydration is well known it is still remarkable that the stabilising effect on a complex protein-like r-VIII SQ is so extensive. However, it should be noted that the stability of r-VIII SQ had been improved to some extent by earlier preformulation work such as providing optimum pH conditions, using nitrogen in the headspace, including calcium and increasing the ionic strength (Fatouros et al., 1997a).

A high concentration of solutes increases the surface tension of a solution but this is not thought to generally hinder the surface adsorption of proteins. If albumin is stabilised by sucrose it might be able to protect r-VIII SQ from surface adsorption (Bagnall, 1977). For example, the r-VIII SQ formulation containing Haemmacel® plus sucrose was not extensively stabilised during agitation (surface adsorption of r-VIII SQ was not hindered), but was significantly stabilised during long term storage (by preferential hydration of r-VIII SQ).

3.2. Surface tension

Measurement of surface tension permits investigation of the interaction between an amphiphilic substance, such as a protein, and an interface (air) and may therefore provide information on conformational changes. The adsorption of a protein at the solution/air interface changes the surface tension of the solution, which can be measured. When a new surface is created, the protein has to reach the surface in order to be adsorbed. This process is limited by diffusion, which creates a lag time; the surface tension decreases exponentially until equilibrium is attained at the newly formed surface. For a large protein like factor VIII, the diffusion is slow and the lag time can be quite substantial, especially if the protein concentration is low.

The behaviour of r-VIII SQ at a newly formed surface and the influence of temperature on this behaviour were investigated using the pendant drop method. The corresponding buffer containing the same formulation but excluding the protein was also investigated. Equilibrium in samples containing r-VIII SQ was not reached for over 1 h, after which the experiment was discontinued (data not shown). A substantial and reproducible decrease in surface tension was attained after 15 min (66.2 ± 1 mN/m after 15 min compared to 65.2 mN/m after 1 h at 20°C). This point was therefore chosen for further study, especially since other difficulties (e.g. evaporation from the drop) occurred if the measurement period was

extended. The buffer alone and water did not show this exponential decrease in surface tension.

The variation in the surface tension of the buffer with temperature was similar to that seen with pure water (Fig. 1C). The presence of r-VIII SQ, however, decreased the surface tension, and this decrease was more pronounced at elevated temperatures (above 20°C). This indicates that at elevated temperatures the conformational flexibility of the protein increases, thereby exposing hydrophobic domains/amino acids that increase the driving force towards the hydrophobic interface. The surface tension in the low temperature range 5–20°C did not indicate conformational changes resulting in increased hydrophobicity (Fig. 1C). Nor did an increase in ionic strength from 18 to 58 mg/ml (1 M) sodium chloride significantly change the surface tension of r-VIII SQ (65.9 mN/m at 20°C). However, the surface tension of the buffer increased slightly, from 72.9 to 75.3 mN/m. This is not unexpected, since osmolytes are known to increase the surface tension of water (Arakawa and Timasheff, 1982).

3.3. Circular dichroism

Alterations in the secondary structure of proteins may be detected in far UV CD (186–260 nm) and alterations in the tertiary structure may be observed by spectral changes in the near UV CD (240–340 nm) (Strickland, 1974; Manning, 1989; Johnson, 1990). The complete three-dimensional structure of plasma-derived or recombinant factor VIII has not yet been determined (Pan et al., 1995). Far UV CD measurements on r-VIII SQ showed mainly a β-sheet structure, but interference from sodium chloride present in the solution was strong below 200 nm. Far UV CD spectra (200–260 nm) were not influenced by temperature in the range 5–55°C, nor by EDTA addition or pH variation, indicating that the secondary structure was largely intact (data not shown).

Spectral changes in the near-UV CD are caused by changes in the chiral environment of aromatic residues and disulphides, thus reflecting alterations in the tertiary structure of proteins (Strickland, 1974). The r-VIII SQ molecule contains several aromatic residues which are seen with CD (75 phenylalanine, 68 thyrosine, 28 tryptophan) and seven disulphides. Near UV CD spectra of r-VIII SQ showed a large negative band around 242 nm, two positive bands at 255–275 and 275–300 nm, respectively, and a negative trough beginning at 300 nm and extending towards higher wavelengths (this remained unchanged in the temperature range 5–40°C) (Fig. 2A). Changes were noted above 45°C, especially at the minimum at 242 nm. At 55°C the spectra indicated major structural changes, although the measurement was probably perturbed by light scattering from the precipitating protein particles.

Differential scanning calorimetry studies have shown that increasing the ionic strength of sodium chloride from 18 to 58 mg/ml improved the thermal stability of the protein (Fatouros et al., 1997a). This correlated well with the results from real-time stability studies. Near UV CD spectra at pH 7 did not reveal any structural changes with increasing ionic strength which could explain the increased stability (Fig. 2B and C). However, at pH 6.3 and 7.7 the spectra, especially around 242 nm, were more perturbed at a low ionic strength (18 mg/ml sodium chloride), indicating structural changes which could lead to a decrease in stability (Fig. 2B). At an ionic strength of 58 mg/ml of sodium chloride a broader pH range (5.8–10) was investigated because precipitation of the protein was inhibited at this concentration of sodium chloride (Fig. 2C and D). The possible structural protection of the protein offered by increasing the ionic strength of the solution could be a combination of preferential hydration and shielding of the induced ionic changes caused by changing the pH. The structural integrity of the molecule seems to be more sensitive to acidic pH according to the near UV CD spectra. This has been confirmed in earlier studies by activity measurements which have shown that a large part of the VIII:C was lost when the pH was below 6 (Österberg et al., 1997).

Near UV CD spectra of r-VIII SQ formulated with some of the macromolecules investigated above indicated only minor interactions with the protein with the exception of Haemaccel® (Fig. 3A). This is in agreement with the notion that

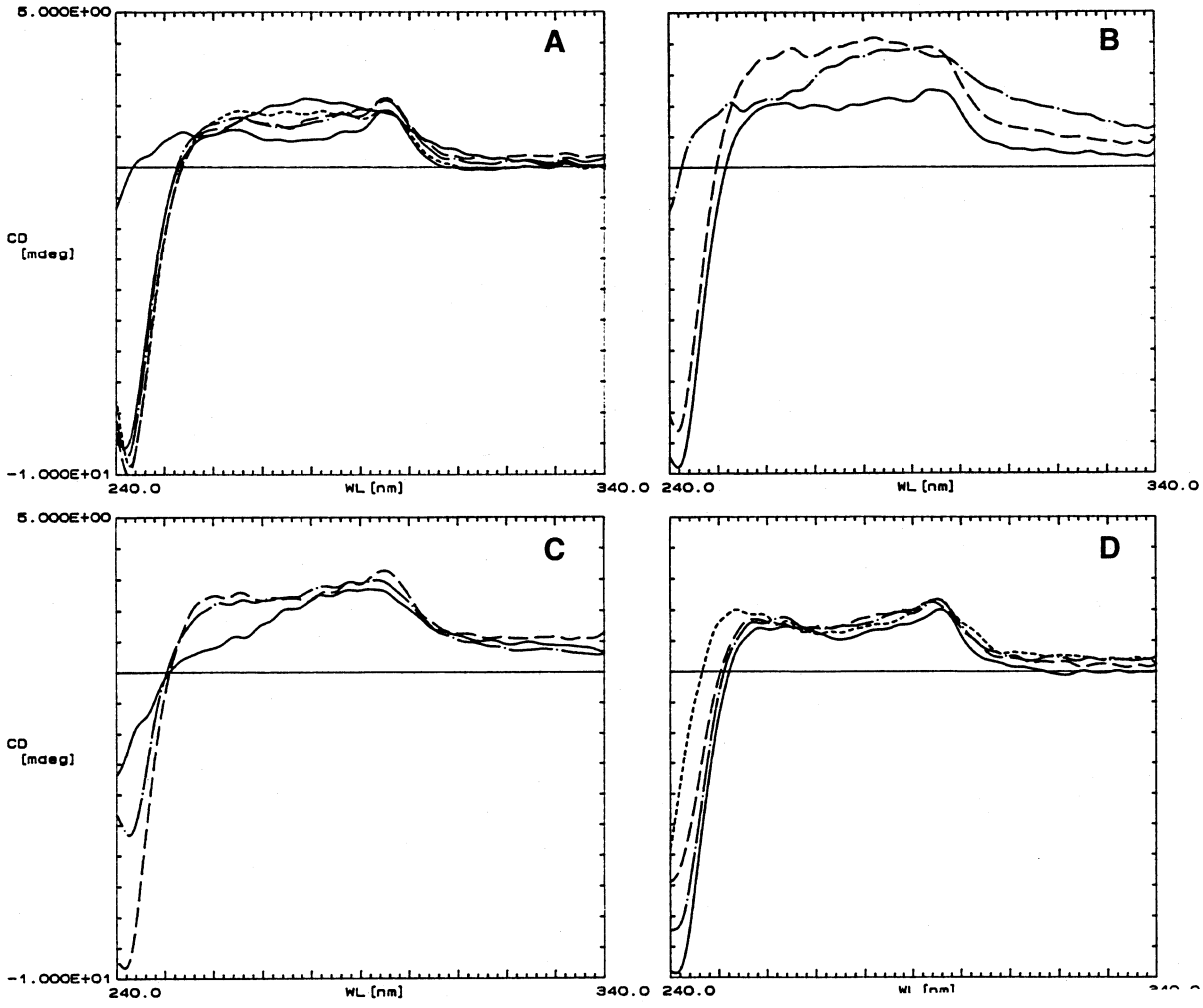

Fig. 2. Near UV CD scan of r-VIII SQ at different temperatures: (A) (— lower curve), 5°C; (– · –), 25°C; (– – –), 35°C; (- - - - - - -), 45°C; and (— upper curve), 55°C; (B) with 18 mg/ml sodium chloride at (– · –), pH 6.3; (—), pH 7; and (– – –), pH 7.7; (C) with 58 mg/ml sodium chloride at (—), pH 5.8; (– · –), pH 6.3; and (– – –), pH 7; (D) with 58 mg/ml sodium chloride at (—), pH 7.7; (– · –), pH 8.4; (– – –), pH 9; and (- - - - - - -), pH 10.

macromolecules which protect proteins during agitation or filtration are thought to do so by being adsorbed at the interfaces in place of the protein rather than by direct interaction with the protein itself (Thurow and Geisen, 1984; Levine et al., 1991).

The amino acid sequence of factor VIII is homologous with those of other metal-binding proteins such as factor V and ceruloplasmin (Pan et al., 1995). It has been demonstrated that the addition of chelating agents results in the separation of the factor VIII light and heavy chains (Andersson et al., 1986). With increasing changes in pH, it is conceivable that local changes in charge may lead to a weakening of the metal ion bridge and/or further conformational changes. Either event could induce chain separation with concomitant activity losses.

There is still some uncertainty regarding the identity of the ion; Ca^{2+}, Mn^{2+} or Cu^{2+} have been suggested (Weiss, 1965; Mikaelsson et al., 1983; Nordfang and Ezban, 1988). Bihoreau et al.

(1994) have shown that factor VIII preparations contain Cu^{2+} but their studies did not exclude the presence of other metal ions. Sudhakar and Fay (1998) have shown that Cu^{2+} serves an auxiliary role as a ligand in factor VIII but that it does not directly mediate intersubunit interaction. Both subunits are implicated in binding copper, but the effects of this binding differs from the interactions observed with Ca^{2+} or Mn^{2+} (Sudhakar and Fay, 1998).

The addition of a chelating agent, 10 mM EDTA, to r-VIII SQ was detrimental to VIII:C (data not shown). The VIII:C loss was partly reversed by the addition of a divalent metal ion. Near UV CD spectra at pH 7 mainly indicated changes around 242 nm that closely resembled the changes seen with a basic or acidic pH (Fig. 3B). The material used in the CD studies contained 4 mM calcium chloride dihydrate from the start; thus, the effect of EDTA was first seen at concen-

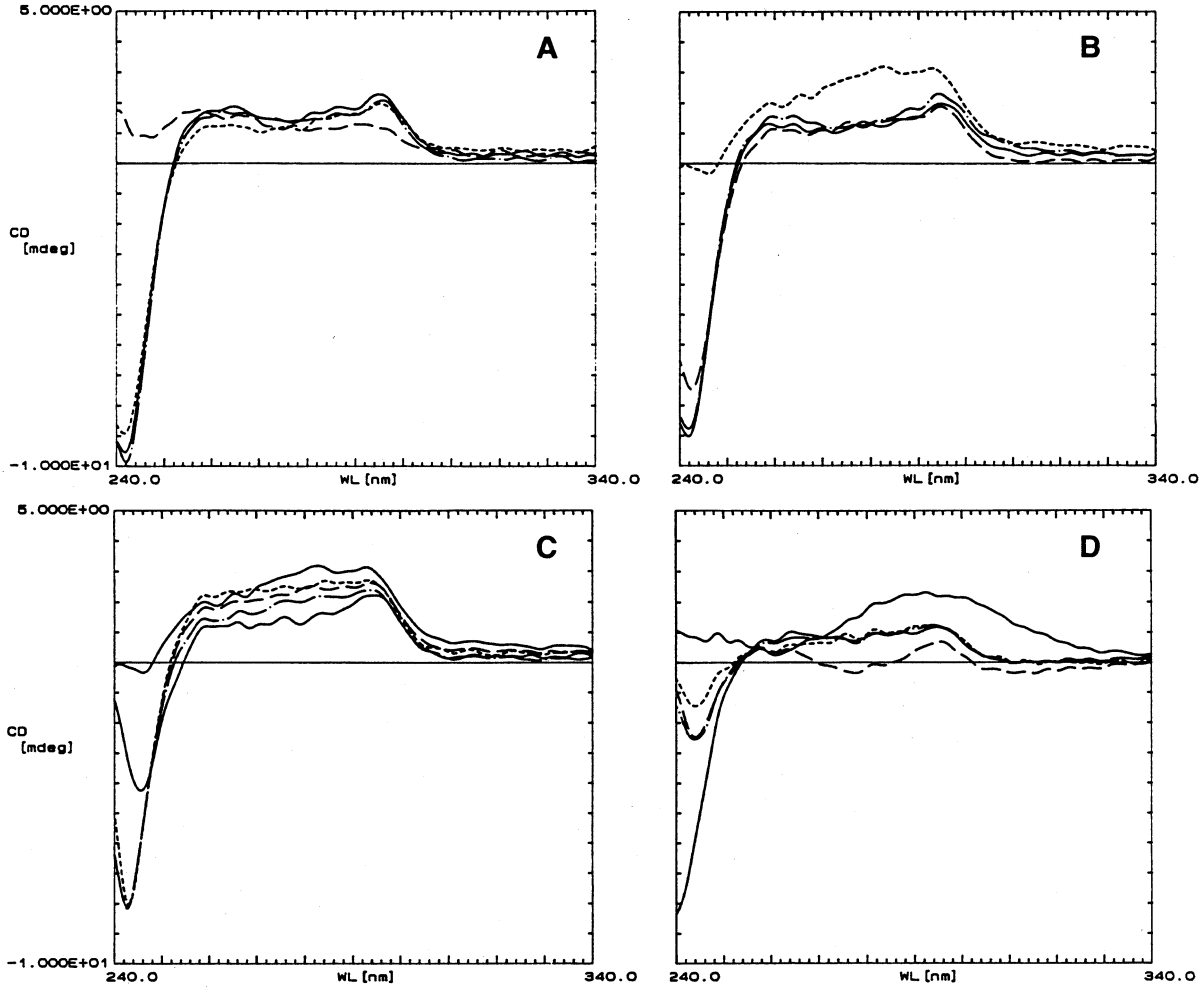

Fig. 3. (A) Near UV CD scans of r-VIII SQ with macromolecular additives: (—), 0.2 mg/ml polysorbate 20; (– · –), 0.2 mg/ml Genapol 80; (– – –), 20 mg/ml Haemaccel; and (- - - - - - -), 300 mg/ml sucrose. (B) Near UV CD scan of r-VIII SQ with EDTA: (—), 0.1 mM; (– · –), 1 mM; (– – –), 5 mM; and (- - - - - - -), 10 mM; (C) with (— upper curve), 10 mM EDTA initially and later additions of 50 mM each; (– · –), Ca^{2+}, (– – –), Sr^{2+}, (- - - - - - -), Mn^{2+}, (— lower curve), Mg^{2+}; (D) with (– · –), 10 mM EDTA initially and later addition of Cu^{2+}; (– – –), 0.01 mM; (- - - - - - -), 0.1 mM; and (— upper curve), 1 mM; and (— lower curve), r-VIII SQ starting material for reference.

trations above this. When investigating the effect of Cu^{2+} on r-VIII SQ, the L-histidine buffer was exchanged for a tris buffer since Cu^{2+} was found to interact with L-histidine and give rise to an artefact spectrum. The artefact spectrum was strongly concentration dependent and was only evident in the range 1:2–1:200 mol/mol Cu:L-histidine.

The effects of 10 mM EDTA on the near UV CD spectra of r-VIII SQ at pH 7 were completely reversible by the addition of an excess (50 mM) of Ca^{2+}, Sr^{2+} or Mg^{2+} ions (Fig. 3C and D). Mn^{2+} partly restored the spectra (Fig. 3C). The addition of 50 mM Cu^{2+}, Zn^{2+} or Fe^{2+} precipitated the protein. Even at low concentrations (1 mM) and without the addition of EDTA, Cu^{2+}, Zn^{2+} or Fe^{2+} perturbed the spectra (data not shown). These results agree well with the stability studies using different divalent ions reported earlier — where Ca^{2+} and Sr^{2+} increased most strongly the stability of VIII:C, followed by Mg^{2+} and Mn^{2+}, while Cu^{2+}, Zn^{2+}, and Fe^{2+} reduced the stability (Fatouros et al., 1997a). The effects of 10 mM EDTA on the near UV CD spectra of r-VIII SQ at pH 6.3 and 7.8 and the effects of a subsequent addition of 50 mM Ca^{2+} were also investigated. The results corresponded to those at pH 7 (data not shown); the other ions were not investigated.

The addition of increasing amounts of various disaccharides and sugar alcohols has previously been found to increase the stability of r-VIII SQ (Fatouros et al., 1997b). Near-UV CD spectra of r-VIII SQ with 300 mg/ml sucrose did not show any change, as seen with increasing concentrations of sodium chloride at pH 7 (Fig. 2A and B, Fig. 3A). It seems that, at the pH optimum, the stabilising effect of the preferential hydration cannot be detected in near UV CD spectra in spite of the large contribution it makes to the protein's thermal and storage stability, and the fact that the near UV CD spectrum is created by aromatic hydrophobic residues interacting with their local environment. Only destabilising interactions which probably stem from direct binding to the protein (preferential interaction) or changed ionic interactions in the protein are detected by CD. Other methods such as DSC and activity measurements must be used to verify the stabilising effect bestowed by the preferential hydration.

Complete structural determination of plasma-derived or recombinant factor VIII has not yet been performed. Nuclear magnetic resonance (NMR) is at present only appropriate for proteins smaller than 30 kDa and this process requires high protein concentrations at which protein solubility may be limited or aggregation may occur (Kelly and Price, 1997). X-ray crystallography requires crystallized samples which are usually difficult to obtain for proteins. Partial information on the conformation of a large protein such as r-VIII SQ may be gained by spectroscopic methods such as CD. There are several advantages associated with spectroscopic methods: little substance is needed, the methods are non-destructive and they measure the protein in solution. However, since the structural information gained is only partial, other analytical methods, e.g. DSC and activity measurements, should be used in combination in order to add some of the missing pieces to the complex puzzle that protein stability presents.

4. Conclusions

CD spectra and surface tension measurements of r-VIII SQ did not reveal any temperature-induced conformational changes in the temperature range 5–20°C; changes were first noted at elevated temperatures. Surface adsorption of r-VIII SQ during agitation was prevented by the addition of a nonionic surfactant. Preferential hydration improved the storage stability of the protein but did not directly prevent its surface adsorption. Keeping the pH at 7, increasing the ionic strength and introducing some divalent metal ions (Ca^{2+}, Sr^{2+} or Mg^{2+}) preserved the structural integrity of the molecule.

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