Estimation of the distance between the divalent cation binding site of des-1–41-light chain-activated bovine plasma protein C and a nitroxide spin label attached to the active-site serine residue

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The paramagnetic effect of Mn^{2+} on the electron paramagnetic resonance spectrum of a nitroxide spin label covalently attached to the active-site serine residue of des-1–41-light chain bovine plasma-activated protein C, and situated at a distance of approximately 1.2 nm from this amino acid, has been utilized to estimate the distance on the enzyme surface between the single Mn^{2+} site and the free electron of the spin label. This distance has been found to be approx. 1.12 nm. A significant paramagnetic effect of Mn^{2+} on the spectrum of this same nitroxide spin label bound to activated protein C (APC) has been found. However, in this case distance calculations are complicated by the existence of a multiplicity of Mn^{2+} sites on APC. If it is assumed that a single Mn^{2+} site is responsible for the paramagnetic effect on the spectrum of the spin label, the interelectron distance on APC would be approx. 0.90 nm.

INTRODUCTION

Protein C (PC) is a plasma glycoprotein with a molecular mass of approx. 54300, which has been purified from bovine (Stenflo, 1976) and human (Kisiel, 1979) sources. The bovine protein is synthesized as a single polypeptide chain (Long *et al.*, 1984) containing 417 amino acids in known sequence (Fernlund & Stenflo, 1982; Stenflo & Fernlund, 1982; Long *et al.*, 1984). However, PC is expressed in plasma as a two-chain protein, as a result of the release of a dipeptide, Lys₁₅₆–Arg₁₅₇, during processing (Long *et al.*, 1984). The light chain of PC contains 11 residues of γ -carboxyglutamic acid, one Asn-linked carbohydrate attachment site (Fernlund & Stenflo, 1982) and one residue of β -hydroxyaspartic acid (Drakenberg *et al.*, 1983). The heavy chain contains three additional Asn-based sites for linkage of carbohydrate (Stenflo & Fernlund, 1982).

In order for PC to function as a serine protease, activation must occur. This is accomplished by cleavage of the Arg₁₄–Ile₁₅ peptide bond in the heavy chain, with release of a tetradecapeptide. This step is catalysed by the Factor X-activating enzyme from the venom of Russell's Viper in the presence of Ca^{2+} (Kisiel *et al.*, 1976), or by thrombin (Kisiel *et al.*, 1977). Activation by this latter protease is substantially enhanced by a cofactor, thrombomodulin, present in a variety of endothelial cells (Esmon *et al.*, 1982). The activated form of PC, APC, is a serine protease that functions as an anticoagulant by virtue of its ability to inactivate, by proteolysis, important cofactors, Factor Va (Walker *et al.*, 1979) and Factor VIIa (Vehar & Davie, 1980), for two separate reactions necessary for clot formation. The active-site Asp, His and Ser residues are located in the heavy chain of APC (Stenflo & Fernlund, 1982; Long *et al.*, 1984).

Isolation of limited proteolytic digestion products of

PC (GDPC) and APC (GDAPC) has greatly aided structure–function studies of these proteins. Both altered proteins lack the 41-residue amino terminus of the parent molecule and contain none of the γ -carboxyglutamic acid residues (Esmon *et al.*, 1983). The number of divalent cation sites is greatly reduced as a result of removal of this peptide. Whereas, bovine PC and APC contain approx. 16 and nine Ca^{2+} -binding sites, respectively (Amphlett *et al.*, 1981), GDPC and GDAPC contain only a single site for this cation (Johnson *et al.*, 1983). A similar situation exists for Mn^{2+} , in that a single binding site is present on GDPC and GDAPC, whereas, at least seven such sites exist on PC (Hill & Castellino, 1987).

This laboratory has demonstrated that cations are necessary for expression of the amidolytic and estero-lytic activities of APC and GDAPC toward synthetic substrates. While monovalent cations are most effective in this regard (Steiner *et al.*, 1980; Steiner & Castellino, 1985a; Steiner & Castellino 1985b; Hill & Castellino, 1986), divalent cations also display this effect by mechanisms independent of the monovalent cation sites (Hill *et al.*, 1987). We are greatly interested in defining the relationships between the monovalent cation sites, the divalent cation sites, and the active site of APC, which along with other kinetic and physiochemical studies of this protein, will illuminate the mechanisms of the unique participation of cations in activation of the catalytic machinery of this serine protease toward low-molecular-mass substrates. For such studies, GDAPC offers a distinct advantage over APC, since the former enzyme possesses only a single divalent cation binding site (Johnson *et al.*, 1983; Hill & Castellino, 1987), most likely a single monovalent cation binding site (Hill & Castellino, 1986), and a single active site. In the present work we have employed this enzyme, along with

Abbreviations used: PC, protein C; APC, activated protein C; GDPC and GDAPC, limited proteolytic digestion products of PC and APC respectively; p-V, 4-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*p*-fluorosulphonylbenzamide; m-V, 3-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*m*-fluorosulphonylbenzamide; m-IX, 3-methyl(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*m*-fluorosulphonylbenzoate; e.p.r., electron paramagnetic resonance.

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paramagnetic probes of the divalent cation site and the active site, in order to attempt to discover the topographical relationships between these latter regions on the enzyme surface.

EXPERIMENTAL PROCEDURES

Proteins

PC was isolated from fresh citrated bovine plasma by modifications (Steiner & Castellino, 1985a) of the procedure of Stenflo (1976), with the final purification by preparative polyacrylamide-gel electrophoresis similar to that described by Kiesel & Davie (1981). APC was prepared by activation of PC with the Factor X-activating enzyme from Russell's Viper, insolubilized to Sepharose 4B (Steiner *et al.*, 1980). GDPC and GDAPC were isolated from limited chymotryptic digests of PC and APC, respectively. The methods employed were essentially those of Esmon *et al.* (1983), with minor modifications. We have described the procedure that we utilize for these preparations (Hill *et al.*, 1987). All proteins exhibited their predicted behaviour on reduced and non-reduced SDS/polyacrylamide gels with < 2% impurities observable. In addition, the concentrations of APC and GDAPC were routinely 85–100% of the total protein present in these preparations, as determined by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate (Chase & Shaw, 1967).

Spin labels

The compounds 4-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*p*-fluorosulphonylbenzamide (p-V), 3-(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*m*-fluorosulphonylbenzamide (m-V), and 3-methyl(2,2,5,5-tetramethylpyrrolidine-1-oxyl)-*m*-fluorosulphonylbenzoate (m-IX), were synthesized using minor operational modifications of the procedures of Wong *et al.* (1974).

For investigation of the amount of spin label bound to the enzymes, a radiolabelled derivative of m-IX was utilized, since this agent offered the simplest synthetic route for radioisotope incorporation. Here, we employed 5 mCi of [³H]LiAlH₄ for the reduction of the carboxylic acid to the alcohol in the synthesis described by Wong *et al.* (1974). After incubation of the spin labelled inhibitor with the enzyme, under the conditions described below, the reaction mixture was applied to a 1 cm × 32 cm column of Bio-Gel P-30, equilibrated and eluted with a buffer consisting of 0.05 M-Tris/HCl, pH 7.4. The protein fraction was collected and pooled. The amount of radioactivity present was determined by liquid-scintillation counting of a measured portion of the sample. The protein concentration was also calculated for this same pool, after determination of the absorbance at 280 nm of a measured portion of the sample ($A_{1\text{cm},280}^{1\%}$ 13.7).

Reactivity of nitroxide spin labels with GDAPC and APC

A total of 0.04 ml of an acetonitrile solution, containing 18.2 mM-p-V, m-V or m-IX, was added to 2.0 ml of a solution consisting of 0.025–0.030 mM-GDAPC or APC, dissolved in 80 mM-Tris/HNO₃, 6.7 mM-CaCl₂/50 mM-TiNO₃, pH 7.4. Because of slow hydrolysis of the inhibitor in aqueous solution, additional portions (0.04 ml) of the spin-labelled reagent were added at

15 min, 30 min and 90 min, followed by incubation for up to 280 min. The reaction mixture was then incubated at room temperature and portions were removed and assayed for residual amidolytic activity at regular intervals. This latter assay was conducted by diluting a portion of the enzyme solution 20-fold, and adding a total of 0.01 ml of this latter solution to a cuvette containing 1.0 ml of a buffer of 0.05 M-Tris/HNO₃/21 mM-TiNO₃, pH 7.4, and the chromogenic substrate, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide (S-2238), at a concentration of 0.11 mM. The rate of release of *p*-nitroaniline at 30° was monitored at 405 nm with a Cary 219 recording spectrophotometer. After four additions of inhibitor, the final acetonitrile concentration was 7.4% (v/v). Control experiments demonstrated that this level of acetonitrile in the absence of inhibitors did not significantly affect the activity of GDAPC or APC.

After the above assays had indicated that sufficient reaction times had elapsed such that the residual activities of the enzymes were less than 10% of the control, the samples were subjected to gel-filtration chromatography, as described in the preceding section, such that the non-bound inhibitors and TiNO₃ were removed. Subsequent to this, e.p.r. spectra were obtained of the spin-labelled enzymes, as described below.

Rotational correlation times, $\tau_{R(p-v)}$, of the spin-labelled inhibitors, when free and when bound to the proteins, were calculated from the e.p.r. spectra, employing the following equation (Knowles *et al.*, 1976):

$$\tau_{R(p-v)} = 6.5 \times 10^{-10} H_0 \sqrt{\left(\frac{h_0}{h_{-1}} - 1\right)}$$

where h_0 and h_{-1} are the heights of the central- and high-field lines of the spectra, respectively, and H_0 is the linewidth of the central line, in gauss (G).

Distance determination by e.p.r.

Theory. The average distance between the free electron of the nitroxide spin label and Mn²⁺ on the surface of the proteins of interest has been estimated by observation of the influence of Mn²⁺ on the e.p.r. spectrum of the nitroxide moiety of the active site-directed inhibitor. The dipolar interaction between the two paramagnetic species results in a decreased peak amplitude in the nitroxide spectrum which is dependent on the sixth power of the distance (r) between the spins. A theoretical evaluation of this phenomenon was first published by Leigh (1970). This theory provides the following equation that describes the linewidth of the e.p.r. spectrum of the observed spin (δH), assuming that the relative geometry of the two paramagnetic species does not change during the lifetime of the spin states, and that the relaxation rate of the metal is fast relative to the magnitude of the spin-spin splitting between the unpaired electrons:

$$\delta H = C(1 - 3 \cos^2 \theta_R)^2 + \delta H_0$$

where δH_0 is the natural linewidth in the absence of a second spin and θ_R is the angle between the applied magnetic field and a vector joining the two spins. The dipolar interaction coefficient (C) is defined as:

$$C = 2\pi g\beta\mu^2\tau_c/(hr^6)$$

where g is the Lande g -value for an electron (2.002), β is the Bohr magneton (9.27×10^{-21} erg/G), μ is the magnetic moment of the paramagnetic ion (7.482×10^3 Bohr mag-

Fig. 1. Inactivation of GDAPC by p-V

The incubation mixtures contained 29 μM -GDAPC in 80 mM-Tris/ HNO_3 /6.7 mM- Ca^{2+} /50 mM-Tl⁺, pH 7.4. At the times indicated by the arrows (●), 40 μl of an 18.2 mM solution of p-V in acetonitrile was added to 2 ml of the enzyme solution, or (x), 40 μl of acetonitrile was added to 2 ml of the enzyme solution. Portions were removed and assayed for activity toward S-2238, as described in the Experimental procedures section.

netons), h is Planck's constant (6.63×10^{-27} erg·s) and τ_c is the correlation time for the dipolar interaction between p-V and Mn²⁺. With several assumptions, Leigh (1970) has calculated the value of C as a function of the relative amplitude of the central peak for systems of nitroxide radicals.

In order to estimate the distance between the free electron of the nitroxide spin label in the active site of the enzymes and Mn²⁺, a procedure similar to that of Cohn *et al.* (1971) has been employed. For evaluation of C , it is necessary to determine the relative amplitude of the central peak of the nitroxide spectrum under conditions where all enzyme molecules are saturated with Mn²⁺. While it is conceptually simple to determine this value, the experiment is not practical to perform, especially with APC at saturation with Mn²⁺, due to the high interference of the Mn²⁺ spectrum with that of the nitroxide spectrum. Therefore, another approach was employed. Here, the spin-labelled proteins were titrated with Mn²⁺ and the nitroxide peak height at each level of Mn²⁺ was determined and plotted against the log[Mn²⁺] (Leigh, 1970). Such graphs are sigmoidal in nature due to the dependence of the peak amplitudes on Mn²⁺ binding equilibria, and are most conveniently analysed by comparison with computer-generated theoretical titration curves. These latter curves allow a best-fit approach to be used to evaluate the K_d for Mn²⁺ binding and the extrapolated maximum relative peak amplitude. With this latter parameter, C was determined from the theoretical graphs of Leigh (1970).

Distance determination by e.p.r.

Experimental. The experimental data were collected employing a Varian E-line Century Series X-band e.p.r. spectrometer, equipped with 100 KHz modulation. Samples were prepared by loading a series of quartz capillary tubes (1 mm internal diameter) with solutions containing identical concentrations of APC, dissolved in

50 mM-Tris/ HNO_3 , pH 7.4. The concentrations of Mn²⁺ in these samples ranged from 0 to 1.7 mM, at a constant level of spin-labelled enzyme (20 μM –44 μM final concn.). Duplicate samples were prepared, identical with each other, except that one set contained Ca^{2+} at 25 mM. Since Ca^{2+} , at this concentration, displaces all Mn²⁺ from the enzymes (Hill & Castellino, 1987), the inclusion of this diamagnetic cation allowed corrections to be free made for the long-range effects of free Mn²⁺ on the e.p.r. spectrum of the bound spin label.

Following preparation of the samples, they were subjected to centrifugation for approx. 1 min in order to remove trapped air pockets from the tubes. E.p.r. measurements were conducted at a microwave power of 2 mW at a frequency of approx. 9.5 GHz. The modulation frequency and amplitude were 100 KHz and 2.0 G, respectively. In order to observe the three-peak e.p.r. spectrum of p-V, the magnetic field was scanned from 3340 G–3430 G over a 15 min period. During the course of all scans, a continuous flow of N₂ was passed over the glass capillary in order to maintain the sample at room temperature.

Since the p-V e.p.r. spectrum is superimposed on the fourth peak of the Mn²⁺ spectrum, the inclusion of increasing Mn²⁺ concentrations resulted in a concomitant increase in the baseline slopes of the p-V spectra. Therefore, the height of the centre peak was measured as the sum of the distances between the baseline and the positive and negative maxima of the peak. These peak heights were then converted to amplitudes relative to the corresponding sample that contained no Mn²⁺. The long-range effects of free Mn²⁺ on the peak heights of the bound p-V spectra were assessed by measuring the peak heights of similar samples that contained 25 mM- Ca^{2+} . Since negligible amounts of Mn²⁺ are bound to the protein under these conditions (Hill & Castellino, 1987), the data provided a standard curve of the relative amplitude as a function of the concentration of free Mn²⁺. The concentration of free Mn²⁺ in samples not containing Ca^{2+} was determined from their Mn²⁺-e.p.r. spectra, or from calculations employing the Mn²⁺ dissociation constants (Hill & Castellino, 1987). The paramagnetic contribution of the free Mn²⁺ was determined from the standard curve, and then subtracted from the total paramagnetic effects observed. Assuming that the effects of bound and free Mn²⁺ are additive, the adjusted relative amplitudes were taken to result from interactions with bound Mn²⁺ alone. This procedure also served to correct for possible interference from dissolved O₂.

RESULTS

Formation and spectral properties of spin-labelled proteins

The enzymes, APC and GDAPC, have been covalently modified with the nitroxide spin labels, p-V, m-V and m-IX. An example of the time course for inactivation of GDAPC with p-V is shown in Fig. 1, as monitored by the loss of amidolytic activity of this enzyme toward the substrate S-2238. Due to hydrolysis of the fluorosulphonyl group in the aqueous buffer, p-V, in acetonitrile, was added repeatedly to the enzyme preparation at the times indicated in Fig. 1. The data show that approx. 98% inhibition of GDAPC amidolytic activity

Fig. 2. E.p.r. spectra of p-V

Spectrum (a): 182 μM -p-V in water, shown for reference. Spectrum (b): 18.2 μM -p-V in 1.0 mM- $\text{Mn}(\text{NO}_3)_2$. Spectrum (c): 50.8 μM -p-V-GDAPC in 100 mM-Tris/HCl, pH 7.4. Spectrum (d): 57.0 μM -p-V-APC in 100 mM-Tris/HCl, pH 7.4.

can be attained at a time of 280 min. During this same time period, a control experiment conducted by addition of acetonitrile, in amounts equal to these present in the above study with p-V, demonstrates that only minor inactivation is found as a result of its inclusion (an 8% activity loss is found at 280 min). Similar results have been observed with APC and with the reagents m-V and m-IX. From studies with [^3H]m-IX, the stoichiometry of incorporation at 98% inhibition of each enzyme has been found to be 1.04 mol of inhibitor/mol of enzyme, suggesting that only the active-site serine residue has been modified.

E.p.r. spectra under a variety of conditions are shown in Figs. 2 and 3. Spectrum (b) of Fig. 2 shows that in the presence of Mn^{2+} , the p-V signal is located on the rising shoulder of the fourth peak of the Mn^{2+} spectrum. When this nitroxide spin label is bound covalently to either APC or GDAPC, a considerable line broadening and concomitant decrease in intensity of the p-V spectra are observed (spectra c and d, Fig. 2). Restoration of the spectrum of the free spin label was observed after addition of guanidine/HCl to the sample, to a final concn. of 6 M. These experiments show that a reduced mobility of the nitroxide moiety occurs when the inhibitor is bound to the protein, and that this effect is dependent upon the native conformation. The question as to whether significant autolysis of the bound spin label

Fig. 3. E.p.r. spectra of nitroxide spin labels

Spectrum (a): p-V in water, shown for reference. Spectrum (b): 51 μM -p-V-APC in 50 mM-Tris/HCl, pH 7.4. Spectrum (c): 51 μM -p-V-APC in 50 mM-Tris/HCl/200 mM-NaCl, pH 7.4. Spectrum (d): 50.1 μM -m-V-APC in 50 mM-Tris/HCl, pH 7.4. Spectrum (e): 50.1 μM -m-V-APC in 50 mM-Tris/HCl/200 mM-NaCl, pH 7.4. Spectrum (f): 52.2 μM -m-IX-APC in 50 mM-Tris/HCl, pH 7.4. Spectrum (g): 52.2 μM -m-IX-APC in 50 mM-Tris/HCl/200 mM-NaCl, pH 7.4.

occurs was addressed by measuring the intensity of the upfield peak of protein-bound p-V as a function of time. The magnitude of this peak did not change during a period of 7 h, indicating that significant mobilization or reduction of the spin label did not occur, at least up to the latest measured time.

As seen in Fig. 3, this same broadening and reduction in amplitude is seen in the lines of p-V, m-V and m-IX, when bound to APC. The presence of 200 mM-NaCl (Fig. 3c, e and g), a concentration known to greatly stimulate the amidolytic activity of APC and GDAPC (Steiner *et al.*, 1980; Hill & Castellino, 1986), does not have an appreciable effect on the shape of the e.p.r. spectrum of any of the forms of spin-labelled APC. Similar results have been observed with GDAPC and the monovalent cation, Ti^+ . The values for the rotational correlation time, τ_R , of the APC-bound nitroxide have been estimated for spin-labelled APC from the relationship described in the Experimental procedures section. Values of τ_R of approx. 2.5 ns for m-V-APC, m-IX-APC and p-V-APC have been found. A similar calculation for p-V-GDAPC revealed a τ_R of 2.4 ns. These values are

Fig. 4. Effect of Mn²⁺ on the relative amplitude of the centre peak in the e.p.r. spectra of p-V bound to GDAPC

The concentration of p-V-GDAPC was 44 μ M in a buffer of 50 mM-Tris/HNO₃, pH 7.4. The samples contained (○) no Ca²⁺, or (●) 25 mM-Ca²⁺. The data were corrected (●) for the contribution of free Mn²⁺ to its paramagnetic effect, as described in the Experimental procedures section.

compared with those of the free spin labels, of 0.53 ns, and of the protein, of 16 ns; this latter value was calculated from the Stokes-Einstein equation, assuming that APC is a globular protein of molecular mass 54300 Da/molecule undergoing isotropic tumbling in the solvent of interest.

Paramagnetic effects of Mn²⁺ on the e.p.r. spectra of p-V-GDAPC and p-V-APC

The distance between the free electron of p-V and the single Mn²⁺ site (Hill & Castellino, 1987) on the surface of GDAPC was estimated by assessment of the dipolar interactions between this free radical and the paramagnetic cation by e.p.r. measurements of the influence of Mn²⁺ on the e.p.r. spectrum of protein-bound p-V. The effects of increasing levels of Mn²⁺ on the relative amplitude of the centre peak in the e.p.r. solution of p-V bound to GDAPC are shown in Fig. 4. Here it is seen that Mn²⁺ causes a decrease in this parameter in both the absence and presence of 25 mM-Ca²⁺, although the extent of the change is substantially greater in the absence of Ca²⁺. Since this level of Ca²⁺ displaces all Mn²⁺ from the enzyme (Hill & Castellino, 1987), it is most likely that the diminished peak heights obtained in its presence are due to long-range effects of non-bound Mn²⁺ on protein-bound p-V. Consequently, the data obtained in the absence of Ca²⁺ have been corrected, as described in Experimental procedures section, for the contribution of free Mn²⁺ to the diminution of the relative amplitude of the central peak of p-V bound to the protein. Thus, the corrected line of Fig. 4 is thought to portray the dipolar interaction between the spin label and Mn²⁺ bound at the divalent cation site of GDAPC. Similar results were obtained in a parallel experiment in which APC was employed.

Fig. 5. Double-reciprocal plot of the change in relative amplitude (from Fig. 4) as a function of the Mn²⁺ concentration

The proteins were (●) p-V-GDAPC and (○) p-V-APC.

Fig. 6. Plot of the corrected relative amplitudes (see Fig. 4) of the p-V-protein e.p.r. spectra against the logarithm of the concentration of Mn²⁺

The proteins were (●) p-V-GDAPC and (○) p-V-APC.

Estimates of the dissociation constants (K_d) for the interactions of Mn²⁺ with p-V-GDAPC and p-V-APC have been made from double-reciprocal plots of the peak height data. Specifically, the inverse of the change in relative amplitude was plotted against the reciprocal of the [Mn²⁺], as shown in Fig. 5. The intersection point of each line with the abscissa was taken to be the negative reciprocal of the [Mn²⁺] that caused a one-half maximal decrease of the p-V e.p.r. spectra, and was assumed to equal $-1/K_d$ for the interaction of Mn²⁺ with these spin-labelled enzymes. The values obtained were $100 \pm 20 \mu$ M and $460 \pm 70 \mu$ M for p-V-GDAPC and p-V-APC, respectively.

The results obtained upon titration of p-V-GDAPC and p-V-APC with Mn²⁺ have been plotted as the corrected relative amplitude versus $\log [Mn^{2+}]$, as provided in Fig. 6. The curves in this illustration were

generated on a micro-computer, from hypothetical maximum relative amplitude and K_d values, to best-fit the experimental points. For p-V-GDAPC, the optimal curve was generated with a K_d of 80 μM and a maximum relative amplitude of 0.84. For p-V-APC, the curve was obtained with a K_d of 460 μM and a maximum relative amplitude of 0.65. These K_d values are in excellent agreement with those determined above and with a separate determination of a K_d of $74 \pm 11 \mu\text{M}$ for p-V-GDAPC, provided from measurements of the levels of free Mn^{2+} , by e.p.r., in mixtures of Mn^{2+} and p-V-GDAPC (Hill & Castellino, 1987). Employing the theoretical curve of Leigh (1970), which relates the maximum relative amplitudes to the dipolar interaction coefficient, C , values of C of 0.59 G and 2.5 G for p-V-GDAPC and p-V-APC, respectively, have been determined.

Correlation times for the interaction of Mn^{2+} and p-V on the protein surface

Calculation of the distance between the two dipolar centres is dependent upon the correlation time, τ_c , for the modulation of the dipolar interaction. This parameter is determined from the following equation:

$$\frac{1}{\tau_c} = \frac{1}{\tau_{R(p-V)}} + \frac{1}{\tau_{R(\text{Mn}^{2+})}} + \frac{1}{\tau_{M(p-V)}} + \frac{1}{\tau_{M(\text{Mn}^{2+})}} + \frac{1}{\tau_{S(p-V)}} + \frac{1}{\tau_{S(\text{Mn}^{2+})}}$$

where τ_R is the rotational correlation time of the bound paramagnetic species, τ_M is the lifetime of the paramagnetic species in the bound site, and τ_S is the electron-spin relaxation time of the spin creating the relaxing field. The values of $\tau_{R(p-V)}$ are calculated from the e.p.r. spectrum of p-V-GDAPC and p-V-APC to be 2.4 ns and 2.5 ns, respectively, as described above. Assuming that the cation acquires the τ_R of the protein, the $\tau_{R(\text{Mn}^{2+})}$ has been calculated from the Stokes-Einstein equation, employing molecular masses of 49400 Da/molecule for GDAPC and 54300 Da/molecule for APC. Its value is 15 ns for Mn^{2+} -p-V-GDAPC and 16 ns for Mn^{2+} -p-V-APC. The $\tau_{M(p-V)}$ for both of these proteins is ∞ , since the p-V group is bound covalently to each protein. The $\tau_{M(\text{Mn}^{2+})}$ values have been obtained from the relationship, $\tau_M = 1/[(K_d) \cdot (k_{on})]$. Employing the K_d values, above, and assuming k_{on} to be diffusion controlled, i.e., $1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Magerum *et al.*, 1978), the $\tau_{M(\text{Mn}^{2+})}$ is calculated as $1.2 \times 10^{-5} \text{ s}$ for p-V-GDAPC and $2.2 \times 10^{-6} \text{ s}$ for p-V-APC. The term, τ_S , is composed of both the spin-lattice ($\tau_{1,S}$) and spin-spin ($\tau_{2,S}$) relaxation times of the electrons. Since $\tau_{1,S} \geq \tau_{2,S}$, a knowledge of $\tau_{2,S}$ will provide a lower limit for τ_S . In cases where it is possible to observe the e.p.r. spectrum of a paramagnetic ligand bound to a macromolecule, an estimate of $\tau_{2,S}$ can be determined from the linewidth (H_0) of that spectrum through the relationship (Bersohn & Baird, 1966):

$$1/\tau_{2,S} = \pi g \beta H_0 / h,$$

where π , g , β and h are the usual constants. Using the experimental linewidths of 2.5 G for p-V-GDAPC and p-V-APC (Fig. 2), the $\tau_{S(p-V)}$ value is 46 ns. Similarly, for the $\tau_{S(\text{Mn}^{2+})}$, a H_0 of 35 G has been determined by measurement of the e.p.r. spectral linewidth of Mn^{2+} when bound to each protein, leading to calculation of a lower limit for the $\tau_{S(\text{Mn}^{2+})}$ of 3.3 ns. Therefore, from the

above equation, we estimate the τ_c values to be 1.2 ns for the dipolar interaction between p-V and Mn^{2+} on the surface of GDAPC, and 1.3 ns for the same interaction on the surface of APC.

DISCUSSION

In order to incorporate a specific probe into a known region of APC and GDAPC, we took advantage of the protease spin labels designed by Wong *et al.* (1974) to prepare these enzymes labelled with a nitroxide radical. All evidence gathered in this report points strongly to the fact that this group is covalently bound to the active-site serine residue. A total of approx. 1 mol of spin label is incorporated/mole of GDAPC and APC, and essentially all enzymic activity is lost at that point. Further, the rate of incorporation of the spin label appears to approximate satisfactorily the loss of amidase activity of these enzymes. Finally, these spin-label reagents were not incorporated by PC.

Before use of the spin-labelled enzymes for evaluation of the paramagnetic effects of Mn^{2+} on their e.p.r. spectra, we needed to consider whether this derivative was stable when bound to the enzymes. There are at least two potential mechanisms by which the spin label may hydrolyse from the protein molecule and complicate the e.p.r. spectra. Hydrolysis of the ester or amide bond could occur within the molecule, resulting in the release of the nitroxide-containing portion with the sulphonyl group remaining bound to the active site of the protein. Also, desulphonylation, wherein the entire reagent is hydrolysed from the protein, could occur. In order to minimize interference from either of these decomposition reactions, the spin-labelled proteins were used immediately upon passage over the gel filtration column. However, in order to evaluate the extent to which either of these degradation reactions could occur, the upfield peak of p-V in a sample of the gel filtered spin-labelled proteins was analysed by e.p.r. at various times of up to 7 h. This experiment was conducted in the absence and presence of catalytic amounts of APC. Although this peak is particularly sensitive to spin-label motility, the peak magnitude did not change over a period of 7 h, an observation that demonstrates that a liberated and freely rotating nitroxide species was not produced with time. In addition, these same samples were tested in order to determine whether enzymic activity had increased in the samples resulting from liberation of the active-site serine residue. No such activity increase was found. The above evidence indicates that the spin-labelled enzymes were sufficiently stable during the experiments.

The shape of the e.p.r. spectra of the nitroxide-labelled macromolecules is very sensitive to the mobility of the nitroxide. For the cases of GDAPC and APC bound to the three spin labels employed in this investigation, the nitroxide moiety is not highly immobilized on these surfaces. This is dissimilar to previous reported studies with trypsin and chymotrypsin bound to these same agents, wherein the bound spin label was much more highly immobilized on these latter enzymes (Berliner & Wong, 1974). The τ_R values for the spin labels in GDAPC and APC are of the order of 2.5 ns, a value which is classified by Knowles *et al.* (1976) as reflecting a weakly immobilized group.

The cation, Mn^{2+} , is an effective paramagnetic probe of the divalent cation sites on APC and GDAPC, since it

produces kinetic effects similar to Ca²⁺ on both enzymes, and since its binding isotherms to APC and GDAPC are similar to those of Ca²⁺. This is more firmly established for GDAPC, since the single Mn²⁺ and Ca²⁺ sites possess mutual displacement properties (Hill & Castellino, 1987). The paramagnetic effect of Mn²⁺ on the e.p.r. spectrum of p-V-labelled GDAPC and APC has been evaluated, a measurement which allows an estimate of the spatial proximity of the free electron in p-V and Mn²⁺. In the case of GDAPC, these measurements reflect the distance between the free electron on p-V and the single Mn²⁺ site of the enzyme. The free electron of the nitroxide group of p-V is approx. 1.2 nm removed from the active-site serine residue to which the spin label is attached. Employing the dipolar interaction coefficient, C , as calculated in the Experimental procedures section, of 0.59 G, and the τ_c of 1.2 ns, as determined in the Results section, a distance of 1.12 ± 0.04 nm between these two centres has been estimated. Since the correlation time that describes the rotational mobility of bound p-V is nearly an order of magnitude shorter than that of the protein, it is apparent that the nitroxide group of p-V is not totally immobilized on the surface of GDAPC. Therefore, the calculated distance is assumed to be the shortest distance between the Mn²⁺ and the free electron of the rotating nitroxide group on the surface of GDAPC. Further, because the correlation time for the dipolar interaction between the free p-V electron and Mn²⁺ is partially dependent upon $\tau_{S(\text{Mn}^{2+})}$ and $\tau_{S(\text{p-V})}$, which themselves are estimates of only the spin-lattice electron relaxation times, the given distances are considered to be lower limits of the true distance between the paramagnetic centre of p-V and Mn²⁺ on GDAPC.

While Mn²⁺ also produces a substantial paramagnetic effect on the e.p.r. spectrum of p-V attached to APC, the electron-Mn²⁺ distances are difficult to calculate since APC contains approx. nine Mn²⁺ binding sites. Interaction of Mn²⁺ with the spin label will therefore be the sum of the contributions of each Mn²⁺ that produces a paramagnetic effect. In order to obtain some estimate of this topographical relationship, we have employed the simplest approach and have assumed that only a single Mn²⁺ site influences the spectral properties of the APC-bound nitroxide. With this rationale, a similar calculation to that for GDAPC, above, leads to an estimated distance of 0.9 ± 0.03 nm for the p-V-free electron-Mn²⁺ distance. Since the interaction is dependent upon r^6 , any other reasonable choice of conditions would not alter the conclusions substantially. For example, if two Mn²⁺ sites were equidistant from the free electron of p-V, their distance from the nitroxide would be approx. 0.98 nm. In the most extreme case, had we assumed that all nine Mn²⁺ sites were equidistant from the active site, a highly unlikely situation, calculations from the observed data would have led to a Mn²⁺-p-V interelectron distance of approx. 1.2 nm. Using another example, if a second Mn²⁺ site was present at a distance of 0.4 nm farther than the nearest Mn²⁺, the given value for the nearest site would be only underestimated by 0.02 nm. Therefore, while it is unlikely that a large uncertainty is introduced in the calculation of the Mn²⁺-p-V-electron distance on the APC surface with the assumption that only a single Mn²⁺ binding site is responsible for the paramagnetic effect observed, the calculated distance is clearly only an approximation of the true distance.

The interelectron distances reported here have been

calculated according to the theory described by Leigh (1970) to describe the rigid-lattice line shape of an e.p.r. signal in a system of two interacting spins. Since our calculations suggest that the nitroxide group of p-V is not totally immobilized when bound to the active site of GDAPC and APC, the assumption that the relative geometry of the two paramagnetic species does not change during the lifetime of the spin states may not be strictly valid. However, Dalton *et al.* (1987) have found that the Leigh approach to distance calculations between a weakly immobilized nitroxide spin label on a thiol residue in membrane-bound D- β -hydroxybutyrate dehydrogenase, and aqueous phase cations, provided a result that was in agreement with an independent measurement of these same distances. This argues for the effectiveness of the Leigh method of estimating distances for non-rigid systems. Furthermore, More *et al.* (1984, 1985) have shown that the relaxation rate of the metal in some Mn²⁺-nitroxide complexes is slow, relative to the spin-spin splitting between the two unpaired electrons, allowing observation of the spin splitting in the e.p.r. spectrum of the nitroxide. Such splittings have not been observed in our experiments. We have placed considerable effort to ensure that all free p-V had been removed from our samples before e.p.r. analysis, and are confident that the effects observed are due to interactions with nitroxide bound to these enzymes.

Finally, the data of Figs. 5 and 6 demonstrate that the K_d values of the Mn²⁺ sites that exhibit the paramagnetic effects of the e.p.r. spectra of GDAPC and APC are approx. 80 μM and 460 μM , respectively. This former value is in excellent agreement with the K_d value for the Mn²⁺-p-V-GDAPC interaction, of approx. 74 μM , as determined by direct measurements of this binding isotherm (Hill & Castellino, 1987). Since the K_d that characterizes the Mn²⁺ site on APC which exhibits the paramagnetic effect on the spectrum of p-V is different from that for GDAPC, it appears as though one of the weak Mn²⁺ sites on APC is situated at least 0.2 nm closer to the spin label than its strong site.

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