Physicochemical Studies of Pregnancy-Specific β_1-Glycoprotein: Unusual Ultracentrifugal and Circular Dichroic Properties†

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ABSTRACT: The molecular properties of native and modified pregnancy-specific β_1-glycoprotein (SP_1) from human placenta were evaluated by sedimentation equilibrium, gel electrophoresis, and circular dichroic measurements. Native SP_1 contained 6.2% *N*-acetylneuraminic acid (NANA), 5.8% galactose (Gal), 13% *N*-acetylglucosamine, 6.5% mannose, and 1.1% fucose but no detectable *N*-acetylgalactosamine. Treatment with mixed exoglycosidases and α-mannosidase removed 79% of the carbohydrate including all of the NANA and Gal. The intensity of the circular dichroic spectrum of SP_1 in the far ultraviolet was quite low with a positive maximum at 235 nm and a negative maximum at 215 nm. The 235-nm band was lost upon treatment with reducing agents or with guanidinium chloride (GdmCl), but not by treatment with neuraminidase. Treatment of SP_1 with neuraminidase, or with mixed exoglycosidases and α-mannosidase, resulted in decreases of the apparent molecular weight obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Neither exposure of SP_1 to GdmCl nor its reduction and alkylation resulted in the appearance of subunits on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The partial specific volume of SP_1 determined experimentally by comparing sedimentation equilibrium profiles in H_2O and D_2O was 0.695 ± 0.007 mL/g. The molecular weight of SP_1 in 6 M GdmCl (in the presence or absence of reducing agents) by equilibrium sedimentation was $42\,300 \pm 400$. In the absence of denaturing agents, SP_1 existed in the form of aggregates (at least as high as trimeric SP_1) that dissociated only slowly upon dilution. The presence of these aggregates may contribute to the reported molecular heterogeneity of SP_1.

A new protein has been recognized recently in pregnancy serum (Tatarinov & Masyukevich, 1970; Bohn, 1971; Lin et al., 1974) and isolated from placenta (Bohn, 1972). Various names have been given to this β_1-glycoprotein, which we shall call here SP_1,[1] abbreviated from the German for "pregnancy protein no. 1". SP_1 is a major placental product. Its blood level rises as gestation proceeds, and at term, the serum concentration is approximately 200 000 ng/mL (Gordon et al., 1977). In normal men or nonpregnant women, by contrast, circulating levels are below 3 ng/mL (Searle et al., 1978).

The role of SP_1 in normal gestation is unknown. Moreover, unlike placental choriogonadotropin, which shares extensive amino acid homology with pituitary lutropin, and placental lactogen, which shares extensive amino acid homology with growth hormone and prolactin, no such pituitary homologue is known for SP_1. Indeed, none of 24 proteins and glycoproteins examined displaced labeled SP_1 from anti-SP_1, even at concentrations 10 000-fold greater than that of the labeled ligand (Kaminska et al., 1979; I. Calvert and S. W. Rosen, unpublished results).

The amino acid composition and overall carbohydrate composition of SP_1 are known (Bohn et al., 1976; Engvall, 1980). Before attempting to determine its amino acid sequence, and in view of recently reported heterogeneity of SP_1 (Teisner et al., 1978, 1979; Shevchenko et al., 1980; Ahmed et al., 1981; Griffiths & Godard, 1981a,b), we thought it would be of interest to examine certain physiochemical properties of the molecule. The results of these experiments are presented here.

Materials and Methods

Materials. SP_1 (lot 13, no. 17) was purified from human placenta (Bohn et al., 1976). Neuraminidase (prepared from *Vibrio cholerae*) was obtained from Calbiochem-Behring Corp. Acrylamide, Coomassie brilliant blue, and *d*-10-camphorsulfonic acid were purchased from Eastman Organic Chemicals. Standard molecular weight markers were obtained from Pierce Chemical Co. and Miles Biochemicals. Dithiothreitol (DTT) was obtained from Sigma Chemical Co. and sodium dodecyl sulfate ($NaDodSO_4$) from Pierce Chemical Co. Iodo[^{14}C]acetic acid was purchased from New England Nuclear. Mixed exoglycosidases, prepared from *Streptococcus pneumoniae* culture medium, were a gift from Dr. Gilbert Ashwell; this mixture (12.8 mg of protein/mL) contained the following enzymatic activities: neuraminidase, 18.6 milliunits/μL; β-galactosidase, 11.4 milliunits/μL; β-*N*-acetylglucosaminidase, 11.4 milliunits/μL; α-fucosidase, trace (Dr. Gilbert Ashwell, unpublished results). α-Mannosidase (1 unit/mL) was obtained from Miles Biochemicals. The combination of exoglycosidases and mannosidase had no detectable protease activity (Rinderknecht et al., 1968; Cheng et al., 1979).

Reduction and Carboxymethylation. The method of Giudice & Pierce (1976) was used, with minor modifications; 150 μg of SP_1 was dissolved in 30 μL of a solution containing 0.5 M Tris, 2% (w/v) EDTA, 8 M urea, pH 8.5, and 27 μg of dithiothreitol. The solution was flushed with nitrogen and incubated for 3 h at 37 °C. Iodo[^{14}C]acetic acid (12.6 μCi/μmol, 190 μg) was added and the mixture incubated for 30 min at room temperature in the dark. One milliliter of 0.01 M ammonium bicarbonate, pH 7.6, was added, and the solution was dialyzed against ammonium bicarbonate and lyophilized.

Treatment with Exoglycosidases and Neuraminidase. SP_1 (1.66 mg) was treated with mixed exoglycosidases according to the method of Cheng et al. (1979). After 4 h, a precipitate appeared in the experimental tube but not in the control tube

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; GdmCl, guanidinium chloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SP_1, pregnancy-specific β_1-glycoprotein; $pdSP_1$, partially deglycosylated SP_1; Tris, tris(hydroxymethyl)aminomethane.

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that contained the enzymes but no SP_1. The incubation was continued an additional 23 h. The precipitate was washed with buffer and with water and dissolved in 6 M GdmCl for equilibrium ultracentrifugation. For carbohydrate analysis, the GdmCl solution was extensively dialyzed against water, and the resulting precipitate was lyophilized. In a separate experiment, 80 μg of SP_1 was incubated with 40 units of neuraminidase alone in 0.05 M sodium acetate, pH 4.6, for 2 h at room temperature followed by 20 h at 4 °C; no precipitate appeared.

Carbohydrate Analysis. Sialic acid was determined by a periodate–resorcinol method (Jourdian et al., 1971). For the determination of hexose, *N*-acetylhexosamine, and 6-deoxyhexose, a micro-scale modification of previously published methods was used (Nilsson et al., 1979; Lohmander et al., 1980) on 70–150-μg samples of glycoprotein.

Sodium Dodecyl Sulfate Electrophoresis. Gels (12–20% linear gradient) were prepared by minor modifications of the method of Laemmli (1970). Electrophoresis was carried out at room temperature overnight (16 h) at 8 mA/gel. The gels were stained for protein with Coomassie brilliant blue. Fluorography was carried out by a standard method (Bonner & Laskey, 1974).

Circular Dichroic Measurements. Circular dichroic spectra were measured on a Cary Model 61 spectropolarimeter equipped with a thermostated cell holder and standardized with *d*-10-camphorsulfonic acid. Spectra were obtained for each sample between 270 and 210 nm at 24 °C. Appropriate blanks were subtracted for each sample, and mean residue ellipticities (deg cm^2/dmol), $[\theta]$, were calculated according to

$$[\theta] = \frac{(\theta)\text{obsd} \times 105}{10lc} \quad (1)$$

where (θ)obsd is the observed ellipticity, 105 is the mean residue molecular weight of the amino acid moieties of SP_1, l is the path length in centimeters, and c is the concentration of protein in grams per milliliter.

Sedimentation Equilibrium. Sedimentation equilibrium measurements were performed on a Beckman Model E ultracentrifuge equipped with a photoelectric ultraviolet scanner and a temperature control system. Double-sector cells with charcoal-filled Epon centerpieces and quartz windows were centrifuged in a six-hole titanium rotor (An-G Ti). The temperature was maintained at 21.5 °C. Rotor speeds, given in the figure legends, ranged from 12 000 to 24 000 rpm. Molecular weights were obtained from the equilibrium profile of concentration vs. radius as described previously (Osborne et al., 1977). Briefly, the ln c vs. r^2 data were fit to eq 2 by

$$Y(x) = \frac{A + Bx + Cx^2}{1 + Dx} \quad (2)$$

using a nonlinear least-squares analysis in order to obtain the least-squares values of A, B, C, and D. In eq 2, Y corresponds to ln c, and x corresponds to r^2. Apparent weight-average molecular weights (M_w^{app}) were then obtained analytically by using eq 3

$$M_w^{app} = \frac{2RT}{\omega^2(1 - \bar{V}\rho)}\left[\frac{B + 2Cx}{1 + Dx} - \frac{(A + Bx + Cx^2)D}{(1 + Dx)^2}\right] \quad (3)$$

where R is the gas constant, T is the absolute temperature, ω^2 is the angular velocity, $\bar{V}$ is the partial specific volume, and ρ is the solvent density. The partial specific volume of SP_1 was determined by sedimentation equilibrium measurements in H_2O and D_2O as described previously (Edelstein & Schachman, 1967).

Table I: Carbohydrate Compositions of Native SP_1 and of Partially Deglycosylated SP_1

	% by weight						
protein	NANA[a]	Gal	GlcNAc	Man	Fuc	GalNAc	total
native SP_1	6.2	5.8	13.0	6.5	1.1	ND	32.6
pdSP_1	ND	ND	5.8	2.6	0.5	ND	6.9

[a] Abbreviations: NANA = *N*-acetylneuraminic acid; Gal = galactose; GlcNac = *N*-acetylglucosamine; Man = mannose; Fuc = fucose; GalNac = *N*-acetylgalactosamine; ND = not detected.

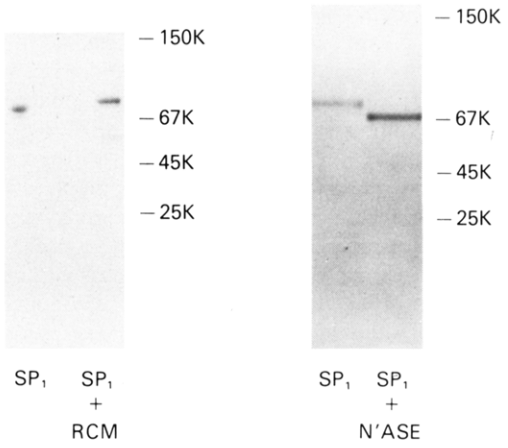

FIGURE 1: $NaDodSO_4$ gel electrophoresis of SP_1, reduced and carboxymethylated SP_1 (SP_1 + RCM), and SP_1 incubated with neuraminidase (SP_1 + N'ASE). Migration positions of standard molecular weight markers are indicated.

Results

Carbohydrate Composition of Native and Partially Deglycosylated SP_1. Results of carbohydrate analysis of native and partially deglycosylated SP_1 are shown in Table I. Data for the native protein are consistent with "complex"-type oligosaccharide chains, N linked to asparagine (Sharon, 1975). *N*-Acetylgalactosamine, a characteristic constituent of oligosaccharide chains O linked to serine or to threonine, was not detected; this result confirms that of Engvall (1980). Treatment of native SP_1 with a mixture of exoglycosidases and α-mannosidase resulted in complete removal of (presumably terminal) *N*-acetylneuraminic acid and galactose and substantial reduction in the amount of *N*-acetylglucosamine, mannose, and fucose. Overall, the enzymic treatment removed 79% of the total carbohydrate.

Sodium Dodecyl Sulfate Gel Electrophoresis. The molecular weight of SP_1, which contains more than 30% carbohydrate by weight (Bohn et al., 1976; Table I), was estimated previously on 5, 7.5, and 10% gels to be 90 000 (Bohn, 1974). We have extended those studies to higher concentrations by using a 12–20% gradient of polyacrylamide. Under these conditions, a dominant band was obtained which corresponded to an apparent molecular weight of 75 000 (n = 12) (Figure 1); a faint band, migrating slightly faster than the dominant band, was seen in the Bohn preparation of placental SP_1 (Figure 1) and in another highly purified placental SP_1 kindly furnished by Dr. Eva Engvall. Reduction and carboxymethylation of SP_1, and treatment with neuraminidase (Figure 1), resulted in apparent molecular weights of 80 000 (n = 1) and 63 000 (n = 3), respectively. Faint bands were detected in the preparation of reduced and ^{14}C-carboxymethylated SP_1, at apparent molecular weights of 74 000 and 51 000, but no

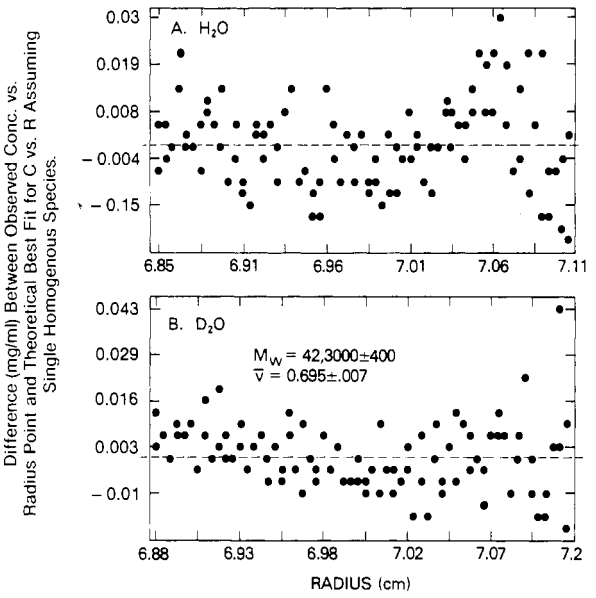

FIGURE 2: Results of sedimentation equilibrium measurements of SP_1 plotted as the difference between experimental concentration profiles and a theoretical profile, assuming a single homogeneous species. The theoretical profile was obtained by a least-squares fit of the data to the following equation: $C(r) = C_m \exp[AM_r(r^2 - m^2)]$ where $C(r)$ is the concentration in milligrams per milliliter at position r in the cell, C_m is the concentration at the meniscus of the cell, M_r is the molecular weight, r is the distance from the center of rotation in centimeters, m is the distance from the center of rotation to the meniscus, and $A = \omega^2(1 - \bar{V}\rho)/(2RT)$ where ω is the angular velocity, $\bar{V}$ the partial specific volume, ρ the solvent density, R the gas constant, and T the absolute temperature. The deviations from experimental data are small and a random function of radius, which is consistent with a single homogeneous species under the conditions studied. The buffer used was 0.01 M Tris, 0.1 M sodium chloride, 0.001 M sodium azide, and 6 M guanidinium chloride. In panel A, the buffer was made up in H_2O, pH 7.4, and in panel B, the buffer was made up in D_2O, pH meter reading of 7.0 or pD 7.4. The rotor speed was 20000 rpm, and the temperature was maintained at 294 K.

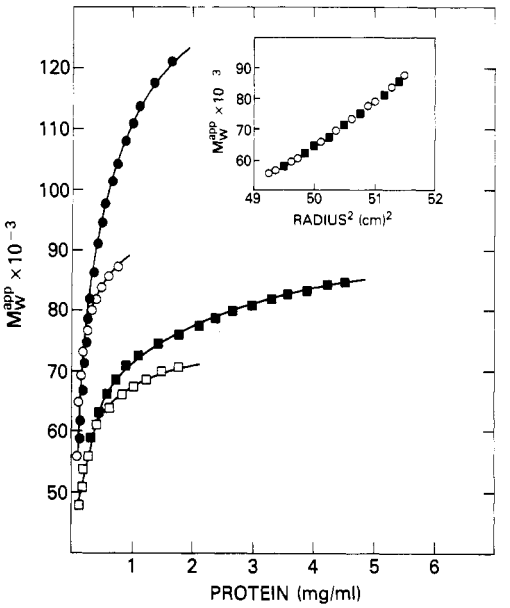

FIGURE 3: Results from equilibrium sedimentation of SP_1 plotted as the apparent weight-average molecular weight vs. the concentration of protein obtained at different rotor speed and different initial protein concentrations. The buffer used was 0.01 M Tris, 0.1 M potassium chloride, and 0.001 M sodium azide, pH 7.4, and the temperature was maintained at 294 K. The apparent weight-average molecular weights were calculated as indicated under Materials and Methods. Initial protein concentrations and rotor speeds, respectively, were the following: 0.27 mg/mL, 12000 rpm (●); 0.27 mg/mL, 18000 rpm (○); 0.45 mg/mL, 18000 rpm (■); 0.58 mg/mL, 24000 rpm (□). *Inset*: Results from equilibrium sedimentation experiments at a single rotor speed plotted as the apparent weight-average molecular weight vs. the square of the distance from the center of rotation for two runs with different initial concentrations of protein. The symbols and other conditions correspond to those given previously for this figure.

Coomassie brilliant blue positive material of lower molecular weight was seen (Figure 1); Engvall (1980) likewise found no evidence for disulfide-linked subunits. Fluorography of the gel also failed to reveal ^{14}C-containing species at less than 51000 apparent molecular weight (data not shown).

Sedimentation Equilibrium. Bohn has reported previously that SP_1 sedimented as two components in the analytical ultracentrifuge, with sedimentation coefficients of 3.0 and 4.6 S (Bohn, 1972). A single component with a sedimentation coefficient of 2.8 S was obtained in the presence of 5 M urea (Bohn, 1972). Estimates of the molecular weight of SP_1 as high as 120000 have been reported on the basis of elution profiles from dextran gel (Sephadex) columns (Bohn, 1972, 1974). In view of these combined results, we evaluated the molecular weight of SP_1 by sedimentation equilibrium. For completeness, we also determined the partial specific volume of SP_1 by comparing the profiles obtained in H_2O and D_2O, as described by Edelstein & Schachman (1967). The combined results, illustrated in Figure 2, gave a partial specific volume of 0.695 ± 0.007 mL/g and a molecular weight (in the presence or absence of reducing agents) of 42300 ± 400. Addition of a reducing agent (DTT, 0.02 M) to the tubes containing SP_1 and 6 M GdmCl did not alter the homogeneity or molecular weight of SP_1 as determined in the denaturant alone (data not shown).

We also measured the molecular weight of $pdSP_1$ by sedimentation equilibrium (data not shown). The analysis was performed in 6 M GdmCl, since $pdSP_1$ was not soluble in the absence of denaturing agents. The partial specific volume used, 0.714 mL/g, was calculated from the published amino acid composition (Bohn et al., 1976) by using the procedure described by Cohn & Edsall (1943) and the correction factor suggested by Hade & Tanford (1967) for preferential binding of GdmCl. The result obtained, 30500 ± 500, was in good agreement with the value obtained for native SP_1, i.e., 42300, given the fact that this molecule contains approximately 30% by weight carbohydrate.

The sedimentation behavior of SP_1 in the absence of denaturing agents is illustrated in Figure 3. With increasing rotor speed and increasing initial concentration of protein, the M_w^{app} at a given concentration of protein decreased dramatically. If SP_1 were an ideal self-associating system, the M_w^{app}, as obtained from equilibrium sedimentation, would be a function of concentration only and independent of rotor speed and initial protein concentration. These data can be accounted for by postulating that the dissociation rate of oligomeric SP_1 is slow compared to the time required to reach equilibrium in the ultracentrifuge (about 12 h in the present study). If this were the case, then the M_w^{app} obtained at a given rotor speed would be a function of the distance from the center of rotation and independent of initial protein concentration. As illustrated in the inset to Figure 3, data at two different initial protein concentrations, obtained at a single rotor speed, overlap one another when plotted as M_w^{app} vs. distance from the center of rotation. Thus, the combined sedimentation equilibrium data are consistent with the formation of oligomers of SP_1 in aqueous solution that dissociate slowly upon dilution.

Circular Dichroism. The circular dichroic spectrum of SP_1 in the far ultraviolet (Figure 4) contained a maximum at 235 nm and a minimum at 215 nm. Treatment with neuraminidase

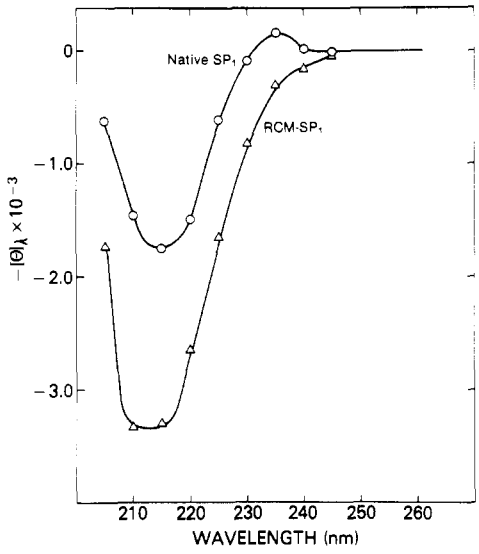

FIGURE 4: Effect of reduction and alkylation on the mean residue ellipticity (deg cm^2/dmol) of SP_1 in the far ultraviolet. The buffer used was 0.01 M Tris, 0.1 M potassium chloride, and 0.001 M sodium azide, pH 7.4, and the temperature was maintained at 24 °C. The circles represent the circular dichroic spectrum of a 0.4 mg/mL solution of unmodified SP_1. The triangles represent the corresponding spectrum after reduction and alkylation. See Materials and Methods for details regarding the modification procedure.

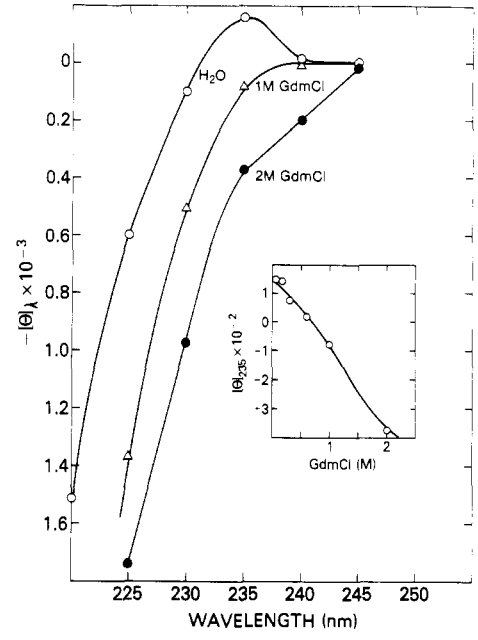

FIGURE 5: Effect of guanidinium chloride on the circular dichroic spectrum of SP_1. The buffer used was 0.01 M Tris, 0.1 M potassium chloride, and 0.001 M sodium azide, pH 7.4, and the temperature was maintained at 24 °C. The open circles correspond to data obtained in the absence of guanidinium chloride, and the open triangles and closed circles represent, respectively, data obtained in the presence of 1.0 and 2.0 M guanidinium chloride. Inset: Plot of the increase in negative mean residue ellipticity (deg cm^2/dmol) of SP_1 at 235 nm as a function of increasing concentrations of guanidinium chloride. Experimental conditions were the same as those given previously for this figure.

to remove sialic acid residues did not affect the circular dichroic spectrum of SP_1 (data not shown). Reduction and alkylation of the sulfhydryl residues resulted in a loss of the 235-nm maximum and an increase in the 215-nm minimum (Figure 4). The maximum at 235 nm was also lost upon treatment of SP_1 with GdmCl (inset of Figure 5), and there was no initial plateau region as is usually found with the GdmCl denaturation of globular proteins.

Discussion

One of the more striking features of the present study is the weak circular dichroic spectrum of SP_1 in the region of peptide bond absorbance. Similar spectra have been reported for certain short polypeptides (Mênez et al., 1980; Cann et al., 1979; Bewley & Birk, 1978; Ikeda et al., 1968; Yoshida et al., 1976) and a few glycoproteins (Alexander et al., 1979; Schmid et al., 1980; Lisowski et al., 1980). It is tempting to speculate that in view of these low intensities SP_1 contains very little organized structure. However, circular dichroic spectra in the far ultraviolet contain contributions from chromophores other than the peptide bond. Thus, any chromophore with a positive ellipticity could counteract substantially the negative ellipticities of α-helical, β-sheet, and random configurations. The increase in negative ellipticity observed upon treatment with GdmCl is also consistent with positive contributions to ellipticity values in the far-ultraviolet region for native SP_1.

Aromatic residues (Woody, 1978) and disulfide bonds (Bewley & Birk, 1978; Woody, 1973; Yoshida et al., 1976) can contribute significant positive ellipticities, at wavelengths as low as 225 nm, to the overall circular dichroic spectra of proteins. However, neither is likely responsible for the 235-nm band in SP_1, since this positive maximum is lost upon treatment with GdmCl as well as with (reduction and) alkylation of the four cysteine residues. Carbohydrate residues may also contribute positive ellipticities in the far-ultraviolet region (Coduti et al., 1977). Although no changes were observed in the circular dichroic spectrum of asialo-SP_1, we were unable to examine the ellipticity of $pdSP_1$, since this material was so insoluble.

The monotonic loss of the 235-nm maximum upon treatment with guanidinium chloride may indicate that this band is strongly dependent upon peptide conformation. Chang et al. (1978) have shown that the β turn can account for strong positive ellipticities with double maxima at 224 and 202 nm, which are comparable in magnitude to the negative α-helical ellipticities at 222 and 208 nm. We have evaluated the circular dichroic spectra of SP_1 in terms of the theoretical reference spectra of helix, random coil, β-sheet, and β-turn configurations proposed by Chang et al. (1978). The results are most consistent with the presence of 32 ± 12% random coil, 40 ± 9% β-sheet, and 28 ± 10% β-turn configurations comprising native SP_1. Of special interest is that the best fit of the data resulted in a contribution of less than 1% for helical configurations. These findings are especially intriguing for SP_1, which contains large amounts of carbohydrate, since it has been suggested that carbohydrate sites in proteins are associated with peptide sequences which favor the formation of β-turn or other turn or loop structures (Beeley, 1977). Data obtained on two additional glycoproteins support this hypothesis. Thus, apolipoprotein $C\text{-}III_2$ and β_2-glycoprotein I, which contain 10% (Osborne & Brewer, 1977) and 16% (Heimburger et al., 1964) by weight carbohydrate, respectively, also have unusual circular dichroic spectra with weak minima in the far ultraviolet (Osborne & Brewer, 1980; N. Lee, H. B. Brewer, Jr., and J. C. Osborne, Jr., unpublished experiments).

The molecular weight of SP_1 in 6 M GdmCl obtained from sedimentation equilibrium measurements was 42 300 ± 400. The higher value of 90 000 estimated previously (Bohn, 1974) and the value of 75 000 obtained by $NaDodSO_4$ gel electrophoresis in the present study are due presumably to the complex behavior of glycoproteins upon electrophoresis in $NaDodSO_4$. We found no evidence for subunits of SP_1 either by $NaDodSO_4$–polyacrylamide gel electrophoresis of the reduced

and carboxymethylated protein or by ultracentrifugal analysis of SP_1 in GdmCl and DTT. Griffiths & Godard (1981a,b) have recently described the separation of SP_1, isolated from pregnancy serum, into two subunits of apparent molecular weight 49 500 and 48 300, as determined by gel chromatography on cross-linked agarose in 6 M GdmCl. They separated the lower molecular weight species into two polypeptide chains of apparent molecular weight 33 500 and 14 800 after reduction with 0.1 M mercaptoethanol and alkylation with iodoacetic acid. Since Griffiths and Godard analyzed SP_1 prepared from serum, and since the details of its purification differed substantially from those of our placental SP_1 preparation, their results and our own are not directly comparable.

Interestingly, in the absence of denaturing agents, SP_1 exists in the form of aggregates that dissociate only slowly upon dilution. The two major components observed in the sedimentation velocity measurements reported previously by one of us (Bohn, 1972) presumably represent monomers and dimers of SP_1. The upper limit of the aggregate molecular weight of SP_1 is not known with certainty. The highest molecular weight found in the present study, 122 000, would suggest the presence of oligomers at least as high as trimeric SP_1.

Teisner and co-workers have reported recently that SP_1 preparations are electrophoretically heterogeneous. A protein that migrated with the α_2-globulins on electrophoresis was found to contain antigenic determinants in common with the classically described β_1-migrating SP_1 (Teisner et al., 1978). Teisner et al. (1979) reported that fractionation of pregnancy serum by gel chromatography and subsequent analysis by rocket immunoelectrophoresis in the presence of poly(ethylene glycol) yielded three peaks that were demonstrated by high concentrations of antiserum to β_1-migrating SP_1. We (Rosen et al., 1979) and Griffiths & Godard (1981a), as well as Teisner et al. (1979), on the other hand, found only a single, symmetric peak when gel-chromatographed pregnancy serum was analyzed for SP_1 by radioimmunoassay. The origin of these additional components is unclear. It may relate to the binding of SP_1 by other serum proteins (Bohn, 1979; Tamsen et al., 1981) or to the effects of reagents used in the preparation or analysis of SP_1 on its tendency to oligomerize. In any event, the formation in aqueous solution, at physiologic pH, of SP_1 oligomers may contribute to the heterogeneity noted by workers in several laboratories.

Acknowledgments

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Effect of Cryosolvents and Subzero Temperatures on the Hydrolysis of L-Leucine-*p*-nitroanilide by Porcine Kidney Leucine Aminopeptidase†

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ABSTRACT: The hydrolysis of L-leucine-*p*-nitroanilide by porcine kidney leucine aminopeptidase in aqueous mixed-solvent systems containing methanol, ethanol, dimethyl sulfoxide, and dimethylformamide has been investigated in the −30 to −23 °C temperature range. At 23 °C and pH* values in the 8–10 range, the enzyme is stable for over 25 h in solutions containing 50% v/v of any of these four cosolvents. Measurements of the tryptophan fluorescence of the enzyme at pH* 9.0 confirm that the enzyme is not denatured under these conditions. K_M increases exponentially and k_{cat} decreases linearly with increasing cosolvent concentration. Methanol, in particular, has a very small effect on K_M. Ultrafiltration experiments demonstrate that there is no dissociation of monomers of the enzyme brought about by the presence of 50% v/v methanol or dimethyl sulfoxide. Preliminary tests with the partition method provide no evidence for an acyl-enzyme intermediate. The effect of pH* on k_{cat} and K_M in 50% v/v methanol is very similar to the effect of pH on these kinetic constants in aqueous solution. Lowering the temperature from 23 to 0 °C does not alter the shape of the pH* profile obtained in 50% v/v methanol. The Arrhenius plot obtained in 50% v/v methanol is linear over the −30 to −23 °C temperature range, and the calculated energy of activation, 8.2 ± 0.8 kcal/mol, is in good agreement with the value of 7.4 ± 0.7 kcal/mol found for the reaction in aqueous solution. Collectively, these data indicate that methanol is the best cosolvent for cryoenzymological studies, that ethanol and dimethyl sulfoxide are also suitable cosolvents, and that the presence of any of these cosolvents at either ambient or subzero temperatures does not perturb the catalytic pathway.

Porcine kidney leucine aminopeptidase (EC 3.4.11.1) is a hexameric zinc metalloenzyme with a subunit M_r of 54 000 (Himmelhoch, 1969; Shen & Melius, 1977; Van Wart & Lin, 1981). It hydrolyzes the N-terminal peptide bond to all L-amino acids except proline and hydroxyproline (Delange & Smith, 1971). In addition, it has both esterase (Delange & Smith, 1971) and thiolesterase (Metrione, 1972) activities. The native enzyme, represented [(LAP)Zn_6−],[1] contains one Zn^{2+} per subunit located at the active (catalytic) site and has an empty regulatory site on each subunit. The enzyme is activated by Mn^{2+} and Mg^{2+} and inhibited by Ni^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+}. These metal ions all exert their influence on the enzyme by binding to the regulatory site. The activating and inhibiting metal ions raise and lower k_{cat}, respectively; K_M is unaltered (Van Wart & Lin, 1981). The Mg^{2+}-activated enzyme is represented [(LAP)Zn_6Mg_6].

Very little information pertaining to the mechanism of action of LAP is presently available. In order to provide such information, we have initiated a series of cryoenzymological experiments to study the hydrolysis of L-leucine-*p*-nitroanilide in aqueous organic solvents at subzero temperatures. Such studies have the potential to resolve elementary steps in the catalytic pathway and yield mechanistic information not attainable under normal conditions (Douzou, 1973, 1977; Fink & Geeves, 1979). In particular, it is possible not only to detect but also to accumulate and stabilize certain intermediates for detailed spectral characterization. Here, we report the effects of cryosolvents and subzero temperatures on the catalytic and structural properties of [(LAP)Zn_6Mg_6]. The enzyme is remarkably stable in the presence of organic cosolvents and is particularly well suited to study by the cryoenzymological approach.

Materials and Methods

Leucine aminopeptidase (type III-CP), obtained from Sigma Chemical Co. as a chromatographically purified $(NH_4)_2SO_4$ suspension, was dialyzed against 5 mM Tris, pH 8, at 4 °C for 2 days and lyophilized. Purified [(LAP)Zn_6−] was prepared by incubation of the lyophilized enzyme with 0.1 mM $ZnCl_2$ for 2 h at 37 °C, followed by affinity chromatography

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[1] Abbreviations: LAP, leucine aminopeptidase; [(LAP)Zn_6Mg_6], metalloleucine aminopeptidase where (LAP) represents the hexameric apoenzyme and the brackets indicate the firm binding of 6 g-atoms each of Zn^{2+} and Mg^{2+} at the catalytic and regulatory sites, respectively; [(LAP)Zn_6−], native leucine aminopeptidase with Zn^{2+} at the catalytic site of each subunit and the regulatory site unoccupied; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid.

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