Biophysical Chemistry, 38 (1990) 1–10
Elsevier

BIOCHE 01416

Study of the structure of streptokinase conjugates with a hydrophilic vinylpyrrolidone copolymer

T.M. Taratina, V.Ye. Potapenko, S.I. Klenin, S.P. Bartoshevich, L.A. Volkova, I.G. Denisov and B.V. Moskvichev

All-Union Research Technology Institute of Antibiotics and Medical Enzymes, 41 Ogorodnikov pr. and Institute of Macromolecular Compounds, U.S.S.R. Academy of Sciences, 31 Bolshoy pr., Leningrad, U.S.S.R.

Received 10 July 1989
Revised manuscript received 21 November 1989
Accepted 24 November 1989

Protein modification; Streptokinase; Reductive alkylation; Conjugate structure; Conformational change

The modification of streptokinase by a synthetic *N*-vinylpyrrolidone copolymer leads to formation of conjugates varying in structures according to the proportions of the components in the reaction medium. Based on data obtained from spectrophotometry, as well as sedimentation and diffusion analyses, it is shown that in the presence of excess protein in the reaction medium, formation of the main chain takes place via the copolymer associated with several protein globules. Under conditions of excess modifier copolymer, either single-site and/or multiple-site bonding is possible for the protein backbone, depending on the molecular weight of the copolymer. One of the models for the conjugates obtained in this manner has been corroborated by small-angle X-ray scattering data. CD spectral analyses has been performed in order to demonstrate that covalent modification does not alter the secondary structure of streptokinase in the conjugate whereas the tertiary structure undergoes local changes in conformation.

1. Introduction

Interest in the modification of proteins by water-soluble polymers has been aroused as a result of the properties shown by the conjugates that are obtained, viz., low toxicity, immunogenicity and allergenization potential, coupled with prolonged effectiveness [1–3]. Conjugates with these features may be recommended as medical preparations. In other cases, conjugates of enzymes and polymers (polyethylene glycol) can function as catalysts in organic media [4,5]. The properties of these conjugates are largely dependent on the modification procedure used [6,7], the chemical nature [8] and molecular weight [9] of the modifier polymer, and the ratio of the concentrations of components of the reaction medium [10,11]. Of particular interest from a scientific and practical point of view are studies on the structure of polymeric derivatives of proteins, the principles involved in their formation and correlations between structure and function.

This article describes the results obtained during the investigation of conjugates of streptokinase, a protein activator of fibrinolysis, and a hydrophilic, linear copolymer of *N*-vinylpyrrolidone. Our objective was the molecular characterization of polymeric conjugates of streptokinase obtained at various ratios of component concentrations in the reaction medium, and the elucidation of their structures using sedimentation and diffusion analyses, small-angle X-ray scattering and CD spectroscopy.

Correspondence address: T.M. Taratina, All-Union Research Technological Institute of Antibiotics and Medical Enzymes, 41 Ogorodnikov pr., Leningrad, U.S.S.R.

2. Materials and methods

A streptokinase protein isolated from avelisin (Germed, G.D.R.), $A^{1\%}_{1cm} = 8.8$ at a wavelength of 280 nm [12], was used in this work. The *N*-vinylpyrrolidone acrylaldehyde diethyl acetal copolymer (Sovial, product of Biolar Research and Product Association to TU 6-09-23-267-83 (Specs)) was activated in an acidic medium. Two copolymer fractions, P_I and P_{III}, were isolated for study of the effect of the molecular weight of the polymeric matrix on the molecular characteristics and specific properties of the enzyme conjugate, via preparative gel permeation chromatography on Sephadex G-200. Copolymer was quantitatively determined using spectrophotometry on the basis of the absorbance of copolymer-iodine complexes at 460 nm [13]. The molar ratio of aldehyde groups in copolymer fractions was assessed by potentiometric assay for the interaction with hydroxylamine hydrochloride [14] and was found to equal 10%.

Chemical modification of streptokinase was effected with the use of polymer fractions as described in ref. 15. Interaction between the ε-amino groups of lysine residues of the protein and the aldehyde moieties of the copolymer yielded *N*-substituted imines, which underwent reduction together with excess aldehyde groups of modified polymers, on reaction with $NaBH_4$. Separation of high molecular weight reaction products from initial components was performed by gel permeation chromatography on Sephadex columns of varying porosity. The chromatograms thus obtained were used to assay the quantity of both modified and native protein as well as that of reacted copolymer and unreacted material.

The enzyme/polymer molar ratios in the conjugates were determined spectrophotometrically and used to evaluate the minimum molecular weight (M) of each conjugate. The number of covalent bonds between modifier polymer was obtained from the loss of free amino groups in the protein. Determination of free NH_2-groups was performed spectrophotometrically, based on the interaction with trinitrobenzenesulfonic acid (Sigma) [17], the experimental data being processed statistically by an Iskra 226 minicomputer. The degree of modification (α) of the enzyme was calculated via three to five parallel determinations as the fraction of NH_2- groups participating in the formation of covalent bonds with the copolymer vs the total present in the activator being titrated. The value of α was used as the basis for the assessment of the number of contacts between the polymer chains and the protein globule. Viscometry involved the use of an Ostwald instrument at $t = 20.0 \pm 0.1°C$, with a period of solvent flow equal to 96 s.

The sedimentation coefficients ($s^\circ_{20,w}$) were determined using an MOM 3180 ultracentrifuge (Hungary) at $20.0 \pm 0.1°C$ and a rotor speed of $50–60 \times 10^3$ rpm. The diffusion coefficients ($D^\circ_{20,w}$) were evaluated with the aid of a polarization diffusiometer [18], calculations being performed via the maximal ordinate and zeroth-order moment methods [19]. The solvent used throughout the study was 0.05 M phosphate buffer (pH 7.5). Sample calculations of M values were according to the Svedberg equation:

$$M_{SD} = \frac{s^\circ_{20,w} \cdot RT}{D^\circ_{20,w} \cdot (1 - \rho_0 \bar{v})} \quad (1)$$

where $s^\circ_{20,w}$ denotes the sedimentation coefficient, $D^\circ_{20,w}$ the diffusion constant, $R = 8.314 \times 10^7$ erg degree^{-1} mol^{-1} is the universal gas constant, T the absolute temperature (here $T = 293$ K), ρ_0 the solvent density ($\rho_0 = 1.004$ g cm^{-3}) and $\bar{v}$ the specific partial volume. The values of the specific partial volume $\bar{v}$ used for streptokinase and the copolymer were taken from published data [20,21], those for the conjugates being determined via the summation rule on the basis of the molar ratios of the components.

Small-angle X-ray scattering curves for solutions of both modified and native enzyme were obtained on a DRON-1 diffractometer complete with a small-angle Kratky camera. Scattered radiation was recorded on an SRS-1 scintillation counter and SSD counting device. CuK_α radiation ($\lambda = 1.54$ Å) was employed, being Ni-filtered via an amplitude discriminator. All sample measurements were carried out in 0.1 M $NaHCO_3$, using cells of 1.4 nm thickness with 0.015 mm thick mica windows. Plots of the scattering intensity (J) were constructed based on a range of scattering

angle ($2v$) of 6.5 minutes of arc to 1°. The radius of gyration of the macromolecules (R_0) was determined from Guinier plots [22]. Small-angle X-ray scattering curves were taken from the values of the finite dimensions of the incidence and collimation slits. Selective calculation of point-like collimation showed that the effect of correction for collimation on R_0 did not exceed 10% of the measured value without such correction. CD spectra were recovered over the range 200–310 nm of wavelengths on a mark III dichrograph (Jobin-Yves, France), at protein concentrations of 0.3–1.0 mg/ml, with instrumental sensitivities of $(1-2) \times 10^{-6}$ A/mm, time constant of 2–5 s and scan speed 0.2–0.5 nm/s in 1.0–2.0 cm quartz cells (within the absorption range of aromatic amino acids, 250–310 nm) and 0.01–0.05 cm quartz cells (absorption for peptide bonds, 200–250 nm). The values obtained are expressed in units of molecular ellipticity $[\theta]$ referred to the average of the amino acid residues of streptokinase as given by the equation [23]:

$$[\theta] = \frac{3300 M_0 sd}{cl} \qquad (2)$$

where M_0 denotes the average molecular weight of the amino acid residues of the enzyme (115), s the instrumental sensitivity (A/mm), d the difference between the spectrum of the substance being tested and the baseline (in mm), c the protein concentration in the solution (mg/ml) and l the cell length (cm).

3. Results and discussion

Table 1 lists the molar ratios of streptokinase and copolymer fractions P_{III} and P_I in the reaction mixture and properties of the conjugates formed, based on the results from spectrophotometry, as well as their minimum molecular weights as evaluated from the molar ratios of the components and on the basis of no polymer degradation occurring, nor aggregation of conjugates present during the reaction process. Table 2 gives the values of the sedimentation constants, specific partial volumes and molecular weights for the enzyme, P_{III}, P_I and their conjugates formed at various molar ratios of the components.

On comparison of the data obtained from analyses of the enzyme-copolymer fraction P_{III}/conjugates listed in tables 1 and 2, respectively, at equimolar ratios of enzyme to polymer in the reaction medium, two types of conjugates appear to be formed which contain equimolar ratios of

Table 1

Preparation conditions and some of the properties of streptokinase-copolymer fraction P_{III}/P_I conjugates

Copolymer fraction M	Conjugate sample	Reaction mixture		Conjugate characteristics			
		NH_2/COH (mol/mol)	SK/P (mol/mol)	SK/P (mol/mol)	Degree of modification	Number of covalent bonds in conjugate per mol SK	Minimum calculated M of conjugate
	SK-P_{III}(1)						
	zone I	1:0.7	1:1.1	1:1	0.13	3	55000
	zone II	1:0.7	1:1.1	1:1	0.13	3	55000
11000							
(P_{III})	SK-P_{III}(2)	1:3.8	1:5.6	1:2.7	0.38	6	74000
	SK-P_{III}(3)	1:30	1:29	1:8.8	0.50	8	140000
	SK:P_I(4)	1:0.8	5:1	2.6:1	0.31	5	174000
60000	SK:P_I(5)	1:3.8	1:1	1:1	0.38	6	104000
(P_I)	SK:P_I(6)	1:30	1:8	1:2	0.50	8	164000 [a]

[a] The molar SK/P ratio and the molecular weight of the conjugate have been determined with due consideration of the chemically bound copolymer, after mathematical separation of the bound and free copolymer zones.

Table 2

Hydrodynamic properties of streptokinase, copolymer fractions P_{III} and P_I and streptokinase-copolymer fraction P_{III}/P_I conjugates

Sample	$[\eta]$ (dl/g)	$s^{\circ}_{20,w(\times 10^{13})}$ (s^{-1})	($\times 10^7$) ($cm^2\ s^{-1}$)	$\bar{v}$ (cm^3/g)	M_{SD}	Most probable SK/P molar ratio in conjugate
SK	0.120	2.8	6.0	0.74	45000	–
P_{III}	0.067	0.7	10.0	0.84	11000	–
SK-P_{III}(1)						
zone I	–	4.0	2.5	0.76	150000	(1:1)×3
zone II	–	2.3	4.5	0.76	53000	1:1
SK: P_{III}(2)	–	2.9	5.7	0.78	60000	1:2
SK-P_{III}(3)	–	4.5	4.1	0.81	130000	1:8
P_I	0.175	1.8	$D^{\circ}_{20,w}$	0.84	60000	–
SK: P_I(4)	–	4.0	2.9	0.77	150000	2:1
SK: P_I(5)	–	3.4	3.3	0.80	127000	1:1
SK: P_I(6)	–	3.0	3.0	0.82	140000	1:2

the components. The streptokinase-P_{III}(I)-II conjugate eluted in zone II as represented in the gel permeation chromatogram is a conjugate of M_{SD} = 53000, while the enzyme-P_{III}(I)-I species eluted in zone I has a 3-fold greater value, i.e., M_{SD} = 150000.

By comparing the values for the hydrodynamic parameters of the native enzyme and the enzyme-P_{III}(I)-I conjugate, it can be shown that a dense associate of three enzyme-P_{III} pairs is formed in this case, with the protein molecule being surrounded by dense braids of polymer (fig. 1a). The

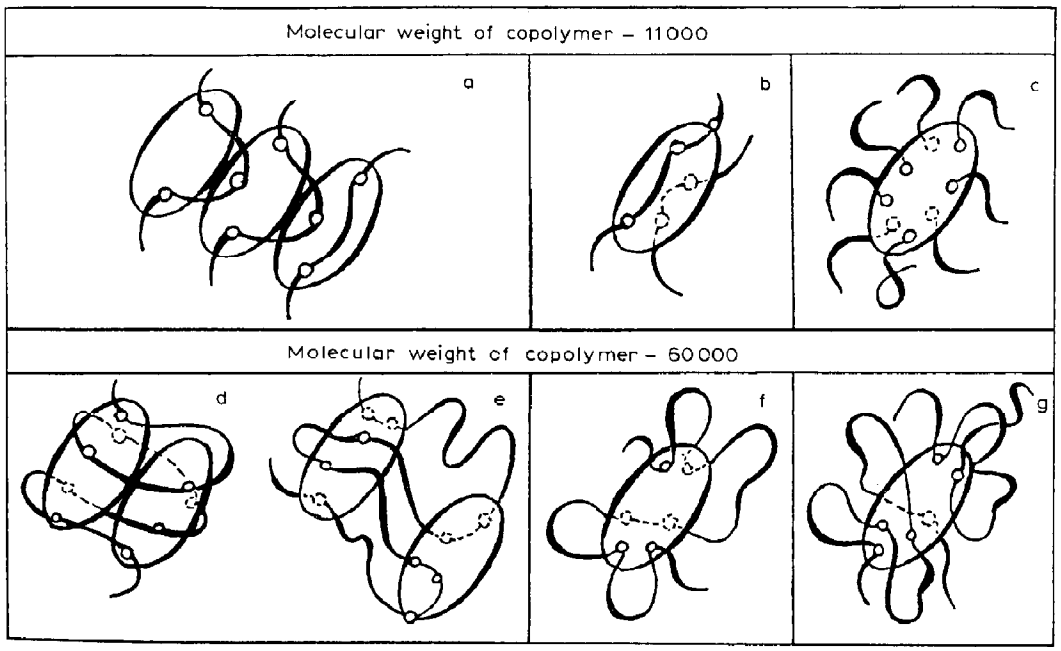

Fig. 1. Schematic representation of the structures of conjugates formed by streptokinase and vinylpyrrolidone-acryaldehyde copolymers, as related to the molecular weight of the copolymer and the ratio of the components.

diffusion coefficient D_0 is known to vary reciprocally with the friction coefficient f averaged over three directions while the sedimentation constant s_0 is directly proportional to the molecular weight and inversely proportional to f. One can readily see that, irrespective of the geometry of the interconnections between individual enzyme molecules, the diffusion coefficient of the enzyme-P_{III}(I)-I conjugate, D_0(c), should be about one half of the value for the enzyme, D_0(sk):

$$\frac{D_0(\text{sk})}{D_c} \sim \frac{1}{f_{sk}} \cdot \frac{2f_{sk}}{1} = 2 \qquad (3)$$

while the sedimentation constants S_0(c) and S_0(sk) must differ about 1.5-fold in value (sk/SK both denote streptokinase):

$$\frac{S_0(\text{c})}{S_0(\text{sk})} \sim \frac{M_c}{f_c} \cdot \frac{M_{sk}}{f_{sk}} \sim \frac{M_c}{2f_{sk}} \cdot \frac{M_{sk}}{f_{sk}} = 1.5 \qquad (4)$$

Comparing S_0, D_0 and M_{SD} for streptokinase and the enzyme-P_{III}(I)-I conjugate, we conclude that the proposed model assuming a structure of the (enzyme-P_{III}) $\times m$ type, with $m = 3$, is capable of satisfactorily describing the hydrodynamic properties of this conjugate.

Regarding the SK-P_{III}(I)-II conjugate, analysis of the hydrodynamics data allows one to draw the conclusion that the conformation of the enzyme has a looser conformation in the conjugate, however, a detailed account of the structural features must await further investigation.

When the protein content of the reaction medium is substantially lower than the amount of modifier polymer, practically all of the protein reacts with the copolymer. With increasing molar excess of modifier both the fraction of reacting amino groups in the enzyme component and the copolymer content of the conjugate increase. Thus, we observed that, for an increase of 5.6 to 29 in molar excess of P_{III} in the reaction medium, the enzyme/P_{III} ratio in the conjugate changed from 1 : 2.7 (2) to 1 : 8.8 (3) as shown by the spectrophotometry data (table 1), or from 1 : 2 to 1 : 8 (mol/mol) on the basis of the results obtained via sedimentation and diffusion analyses (table 2). The number of chemical bonds linking a protein molecule with one copolymer chain falls from 3 to 1 in this case. Hence, under our experimental conditions, an appreciable molar excess of copolymer results in a conjugate with a structure of the type SK-$(P_{III})_n$, where $1 < n < 8$. It is probable that higher values of the copolymer molar excess will not increase the amount of polymer component of conjugates since, the maximum number of amino groups found to be modified in our studies was 8.

It should be noted that the addition of a small amount of polymer chains to a protein globule ($n = 2$), when each P_{III} chain is linked with the SK molecule via several covalent bonds, does not increase the size of the SK-P_{III}(2) conjugate to a substantial degree as compared to the native protein. In fact, their diffusion coefficients are very similar in value (table 2), thus providing further evidence in favour of our assumption that where P_{III} forms three covalent bonds with one molecule of protein, it covers the SK in a dense braiding, without forming any loops. The structure of this type of conjugate is represented schematically in fig. 1b. Conversely, when the modifier polymer P_{III} is connected to the protein molecule at just one point ($n = 8$), the increase in particle size is quite considerable (table 2 and fig. 1c).

We shall now deal with the case where SK is modified with a copolymer of 60 kDa. With SK present in the reaction medium in molar excess, a conjugate is formed with a molar ratio of SK to P_I equal to 2.6 : 1. The molecular weight calculated from this ratio is 174 000, correlating well with that of the conjugate as determined from the results of the sedimentation and diffusion analyses ($M = 150\,000$). Thus, one copolymer macromolecule is linked to an average of two protein globules, with the polymer matrix forming five covalent bonds with each protein molecule.

The structure of this type of conjugate can be modelled in two ways: (i) as a particle comprising two protein globules around which a copolymer molecule is so densely wound that the increase in size, though not in mass, due to P_I can be ignored (fig. 1d); (ii) as a looser formation of two protein globules with a copolymer spacer or interlayer in between, the copolymer forming loops between the bond sites on the protein (fig. 1e). On comparison of the molecular parameters of the conjugate and SK, the second model appears to be

preferred, in which the polymeric spacer must be approximately equal in size to the globule (cf. conjugate SK-P_{III}(I) – zone I). In fact, with the first model being the likelier proposition, the diffusion coefficient of the conjugate and that of globule would differ by a factor of 1.6 only, and the sedimentation constants by a factor of 2.0.

When an equimolar ratio of components is present in the reaction medium, the process of protein-to-copolymer bonding reaches completion. This is also corroborated by the results from the sedimentation and diffusion analyses (table 2 and fig. 1f).

For a molar excess of modifier polymer P_I in the reaction medium ($SK/P_I = 1:8$), a conjugate is formed with an SK/P_I ratio equal to 1:2, which correlates with the value obtained for the molecular weight: $M_{SD} = 140\,000$. In this case, the conjugate exists in the form of a streptokinase molecule to which two chains of copolymer P_I are attached. On considering the equilibrium flexibility of the copolymer chain and the fact a protein globule forms four bonds with each copolymer chain, one may assume that copolymer loops are formed between the bond sites on the protein globule (fig. 1g). The solution behavior of such a conjugate represents the closest approximation of the behaviour of a copolymer of identical molecular weight. Thus for a copolymer with a molecular weight of 140 000, the diffusion coefficient is calculated to be $D^{o}_{20,w} = 3.2 \times 10^{-7}$ cm^2 s^{-1}, and that for the SK-P_I(6) conjugate amounts to $D^{o}_{20,w} = 3.0 \times 10^{-7}$ cm^2 s^{-1}. The same trend can be observed for the sedimentation constants: $s^{0}_{20,w} = 2.9 \times 10^{-13}$ s^{-1} for a copolymer with a molecular weight of 140 000, $s^{0}_{20,w} = 3.0 \times 10^{-13}$ s^{-1} for the SK-P_I(6) conjugate. The structure of this conjugate formed by protein covered by copolymer loops may be approximated by a statistical, flexible-chain copolymer coil. The same type of conjugates was obtained on modification of terrilitin with a vinylpyrrolidone-acrylaldehyde copolymer present in an excess [21].

One of the above models, obtained with a molar excess of copolymer in the reaction medium, was studied using the small-angle X-ray scattering technique. The modifier employed was a copolymer with a value of the molecular weight corresponding to the mean of the values for the two fractions selected, i.e., 35 000. The SK/P ratio in the conjugate resulting was 1:2.6, based on spectrophotometry.

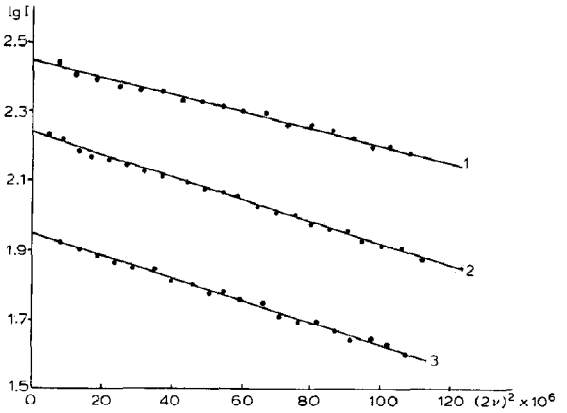

Fig. 2. X-ray scattered intensity curves for native streptokinase at different concentrations: 1, 2.8%; 2, 1.4%; 3, 0.7%.

Fig. 2 presents small-angle X-ray scattering plots on Guinier coordinates of log I vs $(2\nu)^2$ for native SK solutions of differing concentrations. It is readily observed that the experimental points for each solution correspond closely to a linear relationship. This is indicative of uniformity in both the electron density of the scattering and of particle size. The slope of the scattering curves served to determine the radius of gyration R_0 for various protein concentrations. For native SK, extrapolation to zero concentration (fig. 3) gives $R_0 = (40 \pm 2)$ Å. A decrease in R_0 with increasing streptokinase concentration is associated with intermolecular interference at low scattering angles [24].

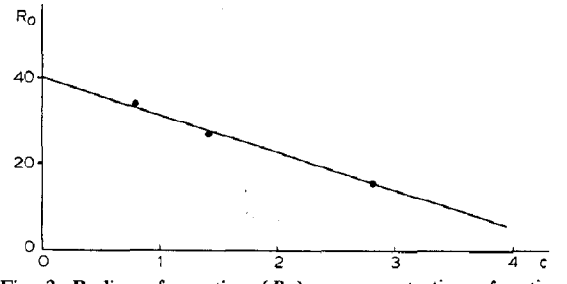

Fig. 3. Radius of gyration (R_0) vs concentration of native streptokinase.

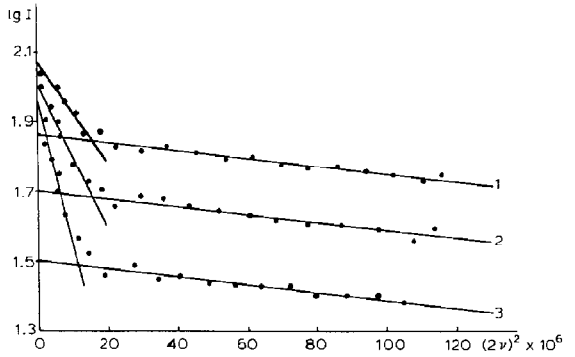

Fig. 4. X-ray scattered intensity curves for modified streptokinase at different concentrations: 1, 1%; 2, 0.75%; 3, 0.5%.

Fig. 4 presents X-ray scattering plots on coordinates of log I vs $(2\nu)^2$ for modified streptokinase with a molar ratio of SK/P of 1:2.6, at three conjugate concentrations. The scattering curves for modified SK differ from those of the native enzyme in that they exhibit two rectilinear segments differing in slope. This form of curve demonstrates a lack of uniformity in electron density of the scattering particles and, as shown in ref. 24, may be adequately described by means of a model assuming a dense nucleus coupled with a loose shell. This represents one of the possible models for a copolymer-modified SK (SK_1-P_n).

We shall now evaluate the parameters of the assumed model based on small-angle X-ray scattering data (fig. 4), viz., $R_{0\,tot}$ and $R_{0\,nucl}$: these were determined to be (114 ± 5) and (34 ± 2) Å, respectively (fig. 5). The value obtained for the radius of gyration of the particle nucleus, $R_{0\,nucl}$ = (34 ± 2) Å, is close to that of the radius of gyration for native SK.

The radius of gyration of the entire particle, $R_{0\,tot}$, is correlated with those of the nucleus and the shell in the following manner [24]:

$$R^2_{0\,tot} = KR^2_{0\,nucl} + (1-K)R^2_{0\,shell} \qquad (5)$$

where K is a parameter defining the mass fraction of the nucleus relative to the mass of the entire particle. It may be calculated from the following formula [24]:

$$K = \sqrt{\frac{I_{0\,nucl}}{I_{0\,tot}}} \qquad (6)$$

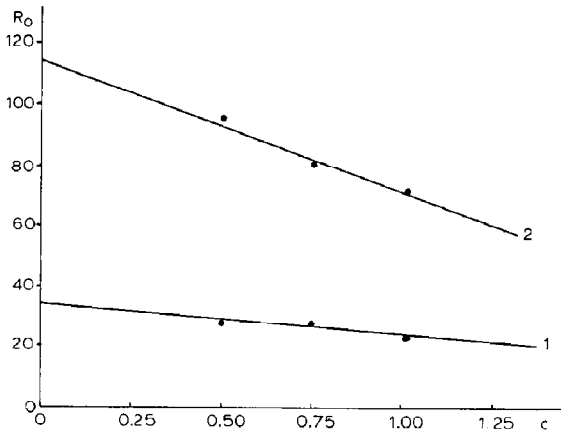

Fig. 5. Radius of gyration vs concentration: for conjugate ($R_{0\,tot}$) and for streptokinase in conjugate ($R_{0\,nucl}$). 1, $R_{0\,nucl}$; 2, $R_{0\,tot}$.

from which the value is found to be $K = (0.28 \pm 0.2)$ (fig. 6).

Using the value of K obtained, the molecular weight and the radius of gyration can be calculated for the polymer shell. In keeping with the selected model, the molecular weight of the nucleus of the scattering particle is assumed to be that of SK, i.e., 45 000 (table 2), the total molecular weight of the conjugate in this case being equal to 160 000. The molecular weight corresponding to the contribution of the polymer shell is then 115 000. Consequently, the stoichiometry of SK/P in such a conjugate is 1 : 3.3, which is in satisfactory accord

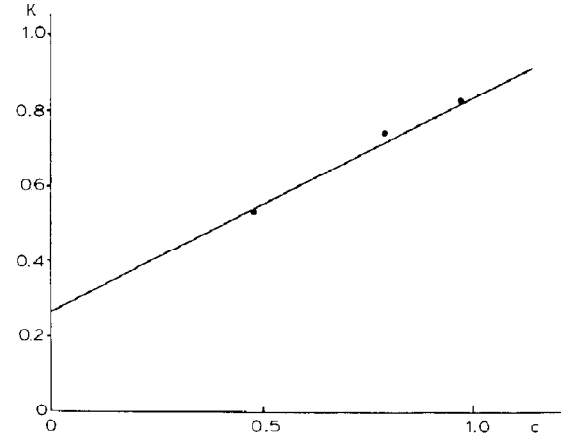

Fig. 6. Determination of the parameter K.

with the data from spectrophotometry (SK/P = 1 : 2.6). The radius of gyration of the diffuse shell, calculated from eq. 5, is $R_{0\,\mathrm{shell}} = (151 \pm 10)$ Å.

Thus, the radius of gyration of the scattering particle nucleus, comparable to that of native SK, and the SK/P ratio in the conjugate, evaluated on the basis of the model, can furnish evidence of the (SK_1-P_n) model being correctly chosen for conjugates with polymer in molar excess over enzyme.

In the foregoing, we discussed the structures and hydrodynamic properties of the conjugates as a whole. Therefore, it would be of interest to study the conformational changes in the protein component of the conjugate in relation to the specific interactions between the atoms and their immediate microenvironment.

Such information can be obtained via investigation of the optical properties of conjugates by recording CD spectra.

Table 3 summarizes the basic characteristics of samples investigated in the absorption regions of the peptide (200–250 nm) and aromatic (250–310 nm) chromophores. It will be noted that all changes in the spectra are consequent upon modification, inasmuch as spectra protein modified without a polymer (P_{III}) or in the presence of a nonreactive polymer with the CHO groups reduced ($P_{III/red}$) correspond with those of native SK. The polymer is optically inactive and does not contribute to the spectra.

It can be seen from table 3 that for all samples studied the value of $[\theta]_{222}$ remains practically

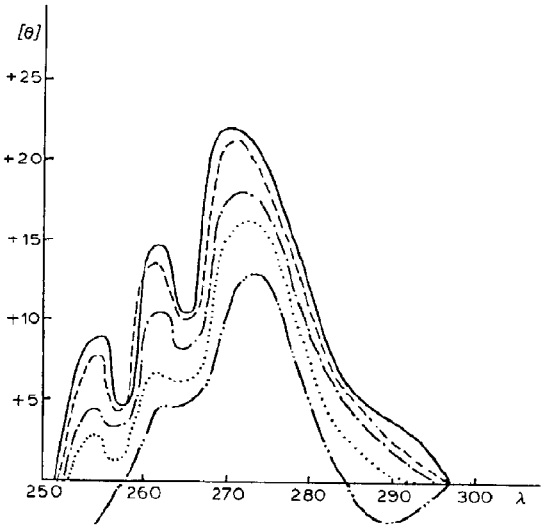

Fig. 7. CD spectra for native and modified forms of SK: (———) native enzyme, (- - - - -) mixture of SK and $P_{III\,red}$ (1 : 5, w/w), (·–·–·) SK-P_{III}(A), (·····) SK-P_{III}(B), (··–··–··) SK-P_{III}(C).

constant (from −3200 to −3400°). Thus, covalent modification resulting in the formation of a conjugate, even when carried out with bound copolymer of higher molecular weight (96 000), does not entail any changes in the secondary structure of the protein at modification levels up to 0.50.

Fig. 7 shows the CD spectra of the samples studied in the aromatic chromophore absorption region. In this case, covalent modification evi-

Table 3

Basic characteristics of the streptokinase-copolymer fraction P_{III} based on CD investigations

Sample	Conjugate characteristics			
	SK/P_{III} (mol/mol)	Total molecular weight of bound polymer ($\times 10^{-3}$)	Degree of modification (α)	$[\theta]_{222}$($\times 10^{-3}$) (degree cm^2 dmol^{-1})
SK	–	–	–	−3.2
Mixture of SK and $P_{III\,red}$ (1 : 5 by wt)	–	–	–	−3.3
SK-P_{III}(A)	1 : 2.7	30	0.38	−3.4
SK-P_{III}(B)	1 : 3.9	43	0.42	−3.3
SK-P_{III}(C)	1 : 8.8	96	0.50	−3.2

dently causes changes to occur in the asymmetrical side group environment of the aromatic amino acids. The molecular ellipticity $[\theta]$ here is reduced somewhat while the fine structure of the CD spectra becomes diffuse. In every case, the extent of the changes increases with greater degree of modification and increasing quantity of bound copolymer. Since the general pattern of the spectra remains unaltered, the above changes may be attributed to local conformational variations in the protein. The trend observed towards decreasing $[\theta]$, as compared to the CD spectrum of native SK constitutes support for the contention that, on the whole, such local conformational changes in copolymer-modified SK lead to definite perturbations of the tertiary structure of the protein. These changes in the specific enzyme structure vary according to the nature of the modifier polymer. For example, on modification of SK with dextrans, instead of the copolymer discussed above, an increase is observed in the molecular ellipticity $[\theta]$ of the conjugate as compared to the native enzyme [25].

The conformational stability of polymeric conjugates of SK to the influence of a number of denaturants may be altered. The conformational stability of the SK component of conjugates with respect to the effect of urea becomes greater with increasing bound copolymer (fig. 8). SDS has the

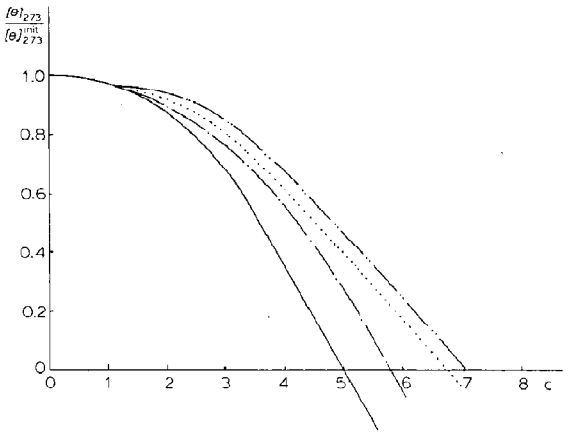

Fig. 8. Variation of $[\theta]_{275}/[\theta]_{273}^{init.}$ in native and modified streptokinases in relation to the molar concentration of urea. (———) Native SK, (·–·–·) SK-P_{III}(A), (·····) SK-P_{III}(B), (··–··–··) SK-P_{III}(C).

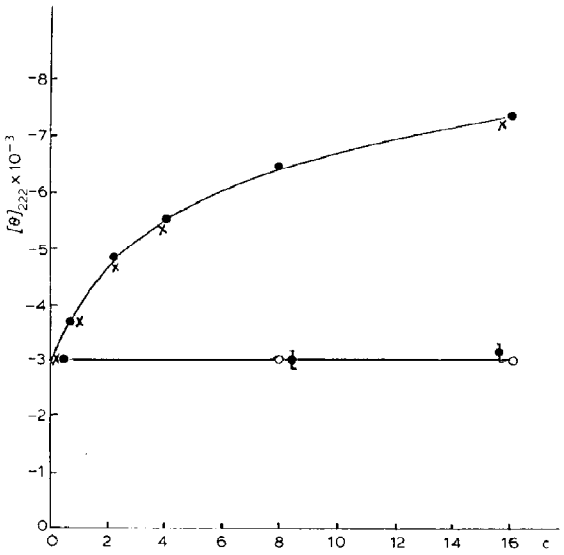

Fig. 9. Variation of $[\theta]_{222}$ in native and modified streptokinase in relation to the molar concentration of SDS: (×) Native enzyme, (●) mixture of SK and $P_{III\,red}$ (1:5, w/w), (●) SK-P_{III}(A), (○) SK-P_{III} (B).

effect of increasing the $[\theta]_{222}$ value in native SK, which may be explained as due to the further structuring of the polypeptide chain of the protein (fig. 9). No ordering of structure would be observed in polymeric derivatives. One may suppose that structuring must be preceded by chain rotation, a contributory factor being the absence of S-S bonds in native SK. As for covalent modification, restriction of the degree of conformational freedom of the protein occurs, preventing it from rotation.

The conformational transition induced by heating is found to occur at temperatures within the range 52–60 °C for both native and modified forms of streptokinase, however, the behavior of samples on renaturation at 20 °C is different, the modified enzyme undergoing renaturation at a slower rate and to a limited extent only.

4. Conclusions

The present experimental results demonstrate the formation of a large variety of molecular structures by the conjugates that result from the reaction of SK with *N*-vinylpyrrolidone-

acrylaldehyde coplymers, the particular structural type being dependent on the ratio of the reactant groups and on the molecular weight of the polymer employed for modification.

Two extreme situations may be noted concerning the conditions selected for the modification reaction:

(1) Protein component present in considerable molar excess (SK > P): in this case, the main chain (backbone) is formed by the copolymer onto which the protein has been grafted. In contrast to synthetic copolymers where bonding to the main chain occurs at a single location, the enzyme protein possesses several chemically reactive sites. Copolymer attachment occurs at several points on the same protein globule, with the copolymer either coating the globule in a fairly dense layer or forming loops together with another globule.

(2) Modifier polymer present in considerable molar excess (SK < P): under these conditions, the backbone is constituted by the protein molecule. The attached copolymer modifies the surface of the protein globule, the degree of modification being related to the molecular weight of the copolymer, and increases the hydrodynamic resistance of the protein during the processes of diffusion and sedimentation.

The conjugate model corresponding to the latter conditions (SK < P) has been corroborated by small-angle X-ray scattering data.

The selected method of modification may be considered to involve relatively mild conditions. The covalent modification of SK by an *N*-vinylpyrrolidone-acrylaldehyde diethyl acetal copolymer does not affect the secondary structure of the protein while its tertiary structure undergoes local conformational changes.

References

1 A.A. Baev, Bioteknologiya (Biotechnology - Russ.), Moscow (1984).

2 Ye.I. Chazov, A.D. Mazaev, J.I. Vironkov and L.A. Suvorina, Ter. Arkh. 9 (1981) 79.

3 T.E. Wileman, R.L. Foster and P.N.C. Elliott, J. Pharm. Pharmacol. 38 (1986) 264.

4 T. Nishio, K. Takahashi, T. Yoshimoto, J. Kodera, Y. Saiko and J. Inada, Biotechnol. Lett. 9 (1987) 187.

5 K. Takabashi, H. Mishimura, T. Yoshimoto, Y. Saito and J. Inada, Biochem. Biophys. Res. Commun. 121 (1984) 261.

6 R.V. Korshak and M.M. Shtilman, Polimery v protsessakh immobilizatsii i modifikatsii prirodnykh soyedineniy (Polymers in the processes of immobilization and modification of natural compounds - Russ.) (Nauka, Moscow, 1984).

7 Vvedeniye v prikladnuyu enzimologiyu (Introduction to applied enzymology - Russ.) (MGU, Moscow, 1982).

8 G.V. Tarasova, V.P. Tikhomirova and M.A. Samartsev, Vysokomol. Soyed. 26 (1984) 496.

9 J. Newmark, A. Abuchowski and G. Murano, J. Appl. Biochem. 4 (1982) 185.

10 N.P. Kuznetsova and G.V. Samsonov, Vysokomol. Soyed. 27 (1985) 2611.

11 T.M. Taratina and B.V. Moskvichev, Khim. Farm. Zh. I (1985) 31.

12 D.M. Kirshenbaum, Anal. Biochem. 68 (1976) 465.

13 G.P. Ivanova, O.A. Mirgorodskaya and B.V. Moskvichev, Vysokomol. Soyed. 21 (1979) 738.

14 R. Roe Harry and J. Mitchell, Jr, Anal. Chem. 23 (1951) 1758.

15 T.B. Tennikova, Ye.F. Panarin, O.A. Mirgorodskaya, G.V. Samsonov and B.V. Moskvichev, Khim. Farm. Zh. 7 (1977) 86.

16 A.L. Ternay, Jr, Contemporary organic chemistry (Mir, Moscow, 1981).

17 R. Fields, Biochem. J. 124 (1971) 581.

18 V.N. Tsvetkov, V.Ye. Eskin and S.Ya. Frenkel, Structura makromolekul v rastvorakh (Macromolecular structures in solution - Russ.) (Nauka, Moscow, 1964).

19 V.N. Tsvetkov, Zhostkotsepnyye polimernyye molekuly (Rigid-chain polymeric molecules - Russ.) (Nauka, Leningrad, 1986).

20 B. Taylor and J. Botts, Biochemistry 7 (1968) 232.

21 T.B. Tennikova, B.V. Moskvichev and G.V. Samsonov, Biokhimiya 45 (1980) 438.

22 A. Guinier and G. Fournet, Small angle scattering of X-rays (Academic Press, New York, 1955).

23 I.A. Bolotina, O.V. Chehov, V.J. Lugauskas and O.B. Ptitsyn, Mol. Biol. (Moscow) 14 (1980) 902.

24 O. Kratky, Prog. Biophys. Biophys. Chem. 13 (1963) 105.

25 V.Ye. Potapenko, I.G. Denisov, T.M. Taratina and B.V. Moskvichev, VI konferentsia po streptoskopii biopolimerov (6th Conference On Spectroscopy of Biopolymers - Russ.) (Kharkov, 1988).