[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Further Physical Chemical Studies on Polypeptidyl Bovine Albumin[1]

BY HAROLD VAN KLEY[2] AND MARK A. STAHMANN

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The electrophoretic mobility, optical rotation and ultraviolet absorption spectrum of bovine albumin modified by the addition of polypeptides of glycine, leucine, phenylalanine, glutamic acid or lysine were studied. Electrophoretic mobility changes were interpreted in terms of increases in charge as measured by *p*H titrations and frictional effects. Optical rotation showed that there was no significant change in the basic structure of the protein after the modification reaction. Ultraviolet absorptivities provided evidence for interactions when polypeptides of lysine were added to the protein; the absorption peaks due to phenylalanine residues were made more apparent in the polyphenylalanyl preparations.

Chemical modification of certain amino acid residues of proteins is one technique used to study the role of specific groups in the properties of proteins.[3] Another method is to study synthetic polypeptides as models of proteins.[4] These two techniques were combined by Stahmann and Becker[5] who prepared polypeptidyl proteins by the addition of amino acid polymers to proteins by reaction with N-carboxyamino acid anhydrides in aqueous buffered solutions.

Studies of some physical and biological properties of polypeptidyl proteins[6-11] and viruses[12,13] have been reported. An extensive study of the immunochemical propreties of polypeptidyl proteins has been undertaken in this Laboratory.[14-17] This investigation was begun to get information on the changes in physical properties brought about by the addition of various polypeptides to bovine serum albumin and to see if the information so obtained could be useful in the interpretation of the immunochemical studies.

Some of the electrochemical behavior of polypeptidyl bovine and rabbit albumins has been reported.[10,11] In this study the electrophoretic experiments have been expanded and the results correlated with the changes in net charge of the protein as determined by *p*H titration.[11] The rotation of polarized light by protein solutions is a function of the constituent amino acids and the contribution of the polypeptide chain configuration, particularly the extent of α-helix formation.[18,19] It was of interest to determine if the addition of polypeptides caused any change in the configuration of the protein chain and also to attempt to establish the configuration of the added polypeptide chains. The presence of amino acid residues with aromatic side chains is responsible for the characteristic ultraviolet absorption spectrum of proteins in the 250–300 mμ range.[18] The added polypeptides were expected to decrease the amount of absorption of the polypeptidyl proteins measured on a weight basis by dilution of the chromophores except when phenylalanine was added; measured on a molar basis there should be no change. An increase which may be interpreted in terms of interactions within the protein molecule was observed with the polylysyl modification in this study.

Experimental

Polypeptidyl Proteins.—The polypeptidyl protein samples were the same as those used in the *p*H titration studies.[11] The dry weight of the samples was determined as described in the previous paper[11]; all concentrations were calculated in terms of the dry weight of the sample.

Preparation of Buffers for Electrophoresis.—Buffers of 0.1 ionic strength were prepared at 25.0° in a constant temperature bath; the *p*H given is that measured at 25.0°.

Electrophoretic Procedure.—Electrophoretic experiments and calculations were carried out as described previously.[10]

Optical Rotation Studies.—Lyophilized protein samples were weighed into volumetric flasks and dissolved in 0.1 *M* KCl to give 1 to 2% (w./v.) solutions. A 2 dm. water-jacketed polarimeter tube was used in a Schmidt and Haensch polarimeter with filtered light of 589 mμ. Temperature was controlled at 20 ± 1°. Observations of rotation were made to 0.01°; an average of 9 to 14 observations was used for calculation. Specific rotations [α] were calculated in the usual manner[20]; molar rotations were calculated by the relationship: [M] = [α] × Molecular weight/100.

Ultraviolet Absorption Spectra.—Solutions prepared for optical rotation studies were diluted with 0.1 *M* KCl to give an absorption at the maximum of 1.0 to 2.0. The ultraviolet absorption spectrum from 350 to 240 mμ was recorded with a Cary Model 11 Recording Spectrophotometer using 1 cm. quartz cells. The slit width was approximately 0.05 mm. over most of this range.

Results and Discussion

The electrophoretic mobility of bovine albumin modified with polypeptides of neutral amino acids is increased at *p*H 8.6[8,10]; the increased net negative

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(2) National Heart Institute Fellow, 1956–1957.

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charge is caused by the substitution of α-amino groups with pK 7.75[21] for ϵ-amino groups with pK 9.8. pH Titrations may be used to determine the number of new α-amino groups formed[6,11] and therefore the approximate increase in net charge. Table I shows the relationship of charge and mobility changes for a series of polypeptidyl bovine albumins.

TABLE I

RELATIONSHIP OF CHARGE AND MOBILITY CHANGES FOR NEUTRAL POLYPEPTIDYL PROTEINS

Protein	Mobility change[a]	Increase in net charge[b]	Mobility change per charge
Polyglycyl$_{232}$ Bovine Albumin A3	−0.31	12	−0.03[c]
Polyglycyl$_{261}$ Bovine Albumin A4	−0.45	18	−0.03[c]
Poly-DL-leucyl$_{46}$ Bovine Albumin F1	+0.63	10	.06
Poly-L-leucyl$_{19}$ Bovine Albumin C2	+ .79	17	.05
Poly-L-phenylalanyl$_{31}$ Bovine Albumin F2	+ .74	6	.12
Poly-L-phenylalanyl$_{36}$ Bovine Albumin F1	+ .74	9	.08
Poly-DL-phenylalanyl$_{48}$ Bovine Albumin C2	+1.58	21	.08

[a] Mobility compared with unmodified bovine albumin in pH 8.6 Na veronal buffer. [b] Measured by pH titration.[11] [c] A decrease was found despite an increase in the net charge.

Both of the polyglycyl bovine albumin preparations showed a decrease in mobility despite an increase in net charge. Glycine polypeptides of average chain length of 19 and 15, respectively, were added to these preparations.[11] The polyleucyl and polyphenylalanyl albumins contained polypeptides of average chain lengths of 2 to 5.[11] In the case of an isolated charged particle, the mobility (u) is equal to q/f[22] where q is the charge and f is a frictional coefficient. The motion of protein molecules migrating in an electrical field is also dependent upon a frictional coefficient and the charge of the molecules. The mobility of polypeptidyl proteins is increased by the charge effect and decreased by the greater frictional effect of the polypeptide chains which may extend out from the surface of the protein molecule. If only short chains are added, an increase in mobility is observed. However, with longer polypeptide chain the frictional resistance to migration is great enough to overcome the charge effect. This suggests that these modified proteins might be useful to study the frictional resistance to migration during electrophoresis.

Each residue of an ionic amino acid can make a contribution to the net charge of the protein; samples prepared by modification with glutamic acid anhydride gave a material for testing this effect. Table II gives the correlation of mobility and charge changes for a series of polyglutamyl albumins at pH 6.3. It shows that the mobility compared to unmodified albumin increased as the net charge was increased by the addition of more glutamic acid residues. However, the mobility change per charge added decreased as more glutamic acid was linked to the protein. Figure 1 is a plot of the increase in mobility compared with the unmodified albumin against the increase in net charge on a series of polyglutamyl albumins. The experimental points are seen to fall on a straight line but this line does not extrapolate to 0 mobility when the change in charge becomes O. The dashed line is the value of 0.2 mobility units per charge calculated by Longsworth and Jacobsen[23] for small changes in charge near the isoelectric point of bovine albumin; the dotted line indicates how the slope might change when the increase in charge is smaller to approach 0.2 as a limiting value. A value of 0.022 mobility units per charge was found in the straight line portion of the graph.

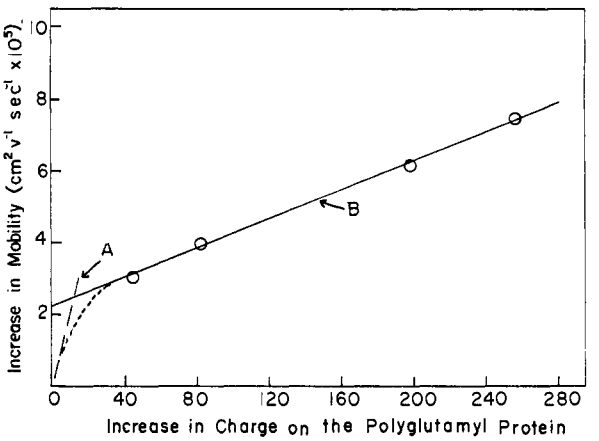

Fig. 1.—Direct relationship between the electrophoretic mobility increase and the increase in charge for polyglutamyl albumins. The increase in mobility over that of the unmodified protein is plotted as a function of the increase in charge. The curve labeled A shows the value of 0.2 mobility units per charge observed by Longsworth and Jacobsen[23] for bovine albumin near the isoelectric point; small changes in the net charge were produced by varying the pH or by the binding of small ions. The curve labeled B is the experimental curve.

TABLE II

RELATIONSHIP OF CHARGE AND MOBILITY CHANGES FOR POLYGLUTAMYL PROTEINS

Protein	Mobility change[a]	Increase in charge[b]	Mobility change per charge
Poly-L-glutamyl$_{41}$ Bovine Albumin C6	+3.04	46	0.066
Poly-L-glutamyl$_{73}$ Bovine Albumin C4	+3.95	81	.049
Poly-L-glutamyl$_{218}$ Bovine Albumin C5	+6.12	199	.031
Poly-L-glutamyl$_{275}$ Bovine Albumin C2	+7.42	257	.029

[a] Mobility compared with unmodified bovine albumin in pH 6.3 Na cacodylate buffer. [b] Measured by pH titration.[11]

Schlessinger[24] in an electrophoretic and titration study of bovine albumin also observed a linear

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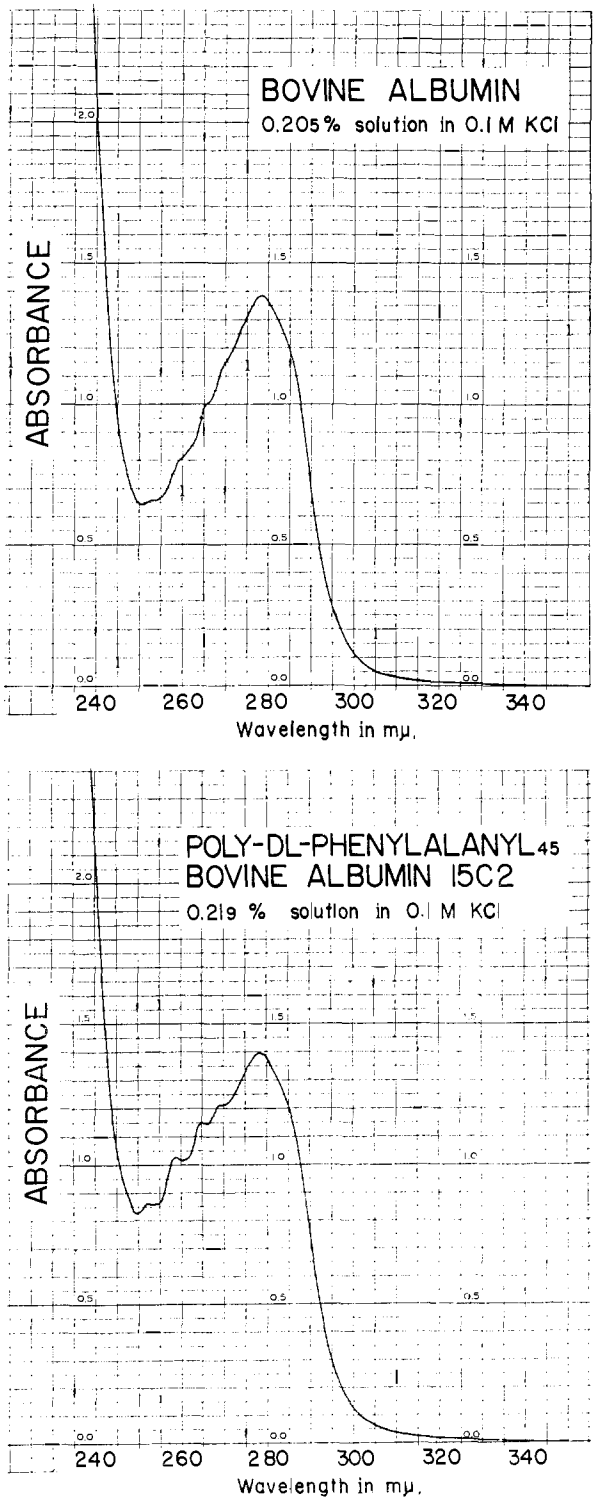

Fig. 2.—Ultraviolet absorption spectrum for bovine albumin (A) and polyphenylalanyl bovine albumin (B).

relationship between mobility and the number of hydrogen or hydroxyl ions bound within limited regions. He further noted that the change in mobility per ion bound was greatest when less than 20 moles of either ion were bound per mole of protein and much less when from 40 to 100 moles were bound. Thus our results and those of Schlessinger suggest that when the net charge on the protein molecule is large, the effect of any single charge in altering mobility is small compared to its effect on mobility when the net charge is small. Structural changes resulting in an increased frictional coefficient were suggested as the probable explanation for the deviations observed by Schlessinger.[24] Similar expansion of the molecule might be expected from the modification by glutamic acid polypeptides carrying many negative charges.

Optical rotation was used to determine if the modification caused any changes in the amount of α-helix in the polypeptide chain of the native protein and to obtain information on the configuration of the added polypeptides. Specific and molar rotations are summarized in Table III. The specific rotation values found for unmodified bovine albumin agree within experimental error with the values reported by Jirgensons[25] and by Yang and Doty.[19]

TABLE III

OPTICAL ROTATION OF POLYPEPTIDYL PROTEINS

Protein	Concn. g./100 ml.[a]	$[\alpha]^{26}D$, degree	$[M]^{26}D$, $\times 10^{-4}$, degree
Bovine Albumin	1.01	−63.3	−4.37
Bovine Albumin	1.44	−61.2	−4.22
Bovine Albumin	1.92	−62.2	−4.29
Polyglycyl$_{261}$ Bovine Albumin A4	0.95	−50.8	−4.27
Poly-L-phenylalanyl$_{31}$ Bovine Albumin F2	1.94	−55.5	−4.08
Poly-L-phenylalanyl$_{36}$ Bovine Albumin F1	1.97	−54.1	−4.02
Poly-DL-phenylalanyl$_{43}$ Bovine Albumin C2	0.88	−56.0	−4.26
Poly-L-glutamyl$_{13}$ Bovine Albumin F1	1.86	−60.6	−4.28
Poly-L-glutamyl$_{41}$ Bovine Albumin C6	2.13	−56.0	−4.16
Poly-L-glutamyl$_{73}$ Bovine Albumin C4	0.68	−55.9	−4.38
Poly-L-glutamyl$_{218}$ Bovine Albumin C5	2.21	−55.0	−5.34
Poly-L-glutamyl$_{275}$ Bovine Albumin C2	1.69	−50.3	−6.20
Polyl-L-lysyl$_2$ Bovine Albumin C$_3$	1.83	−60.4	−4.19
Poly-L-lysyl$_{14}$ Bovine Albumin C1	1.01	−63.4	−4.49

[a] Solvent = 0.1 *M* KCl.

There is no evidence from these optical rotation measurements that the native protein structure has been modified; rotatory dispersion studies would be necessary for a more thorough study of possible denaturation of the protein. In most cases the added polypeptide made no significant contribution to the rotation. The largest change observed in specific rotation was with the polyglycyl sample where the rotation became less negative by approximately 11° but there was no significant change on a molar basis. Approximately 10 γ-benzyl-L-glutamate residues are required for the polypeptide chain to assume the α-helical configuration in organic solvents.[26] If a similar requirement of chain length were necessary for polyglycine to assume the α-

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helical configuration in aqueous solution, the added chains in this preparation were of sufficient size for orientation into a specific configuration. The helical configuration of a polypeptide made up of L-amino acid residues has only one screw sense[27]; in a glycine polymer there are no asymmetric centers to direct the polypeptide into a definite helical screw sense. Therefore if any of the polyglycyl chains assumed a definite configuration such as α-helical, another chain in the configuration of opposite sense must have cancelled the rotation effects.

The only significant changes in molar rotation were found in the polyglutamyl proteins modified with 205 and 259 moles of glutamic acid per mole of protein. The glutamic acid polypeptides would contribute a rotation of approximately -60 to $-80°$,[28] accounting for the small change observed in specific rotation because the protein and polypeptide have similar specific rotations. This would also account for the more negative rotation on a molar basis because both the native protein and the modifying polypeptides contribute to the rotation. All other changes in molar rotation may be considered within the experimental error.

The characteristic ultraviolet absorption spectrum for proteins was observed for the unmodified bovine albumin as illustrated in Fig. 2a. Some shoulders and irregularities from a smooth curve may be seen in the region from 250 to 280 mμ. These shoulders were converted to plateaus or small maxima in the phenylalanyl bovine albumins as shown in Fig. 2b. Table IV contains the calculated 1% and molar absorptivities of the native protein and the polypeptidyl derivatives. The 1% absorptivity of 6.8 for bovine albumin is somewhat higher than the value of 6.60 reported by Tanford and Roberts[29] and by Cohn, Hughes and Weare[30]; this may be because narrower slit widths are possible with the Cary Spectrophotometer as compared with the Beckman DU.

Dilution of the chromophores of the native protein by the added polypeptides accounts for the decrease in the 1% absorptivities; phenylalanine is a chromophore in the ultraviolet but the absorption is at lower wave lengths than 278 mμ as illustrated in Fig. 2b. Molar absorptivities do not show any significant change as a result of the modification reaction.

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The amounts of lysine added during preparation were small compared to some of the other polypeptidyl proteins but nevertheless, a small decrease in absorptivity was expected; however, increases in the 1% absorptivity were found with both polylysyl bovine albumins. Titration of some imidazole and amino groups was apparently shifted to above *p*H 9 in these preparations.[11] The absorption at 278 mμ for proteins is caused mainly by the tyrosyl residues. Based on the shifted titration curve and the increase in absorption, interactions between added lysine amino groups and tyrosyl residues are proposed in the polylysyl bovine albumins.

TABLE IV

ONE PER CENT. AND MOLAR ABSORPTIVITIES AT 278 mμ OF POLYPEPTIDYL BOVINE ALBUMINS

Protein	$a_{1\%}^{278\,m\mu}$	$a_{M}^{278\,m\mu} \times 10^{-4}$
Bovine Albumin	6.7_6	4.6
Bovine Albumin	6.8_5	4.7
Polyglycyl$_{261}$ Bovine Albumin A4	5.8	4.9
Poly-L-phenylalanyl$_{31}$ Bovine Albumin F2	6.4	4.8
Poly-L-phenylalanyl$_{36}$ Bovine Albumin F1	6.3	4.7
Poly-DL-phenylalanyl$_{43}$ Bovine Albumin C2	6.3	4.9
Poly-L-glutamyl$_{13}$ Bovine Albumin F1	6.8	4.8
Poly-L-glutamyl$_{41}$ Bovine Albumin C6	6.4	4.8
Poly-L-glutamyl$_{73}$ Bovine Albumin C4	5.9	4.7
Poly-L-glutamyl$_{218}$ Bovine Albumin C5	4.7	4.6
Poly-L-glutamyl$_{275}$ Bovine Albumin C6	4.7	4.9
Poly-L-lysyl$_2$ Bovine Albumin C3	7.0	4.9
Poly-L-lysyl$_{14}$ Bovine Albumin C1	7.1	5.0

The physical chemical studies indicate that polypeptidyl proteins are good test materials for determination of some of the reactions and interactions of various amino acid residues in proteins. Most of the changes in the physical chemical properties predicted for the various types of modification were found but some unexpected effects were also observed such as the apparent interaction of the added lysine polypeptides with tyrosine residues of the protein. Apparently the structure of the native protein was not greatly changed even by the addition of up to 259 moles of glutamic acid per mole of protein. This agrees with the results from immunochemical studies which show that antisera against the unmodified native protein react with the polyglutamyl albumin and antisera against polyglutamyl albumins react with the unmodified protein.[16]

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