

Jiulin Xia  
Kevin Mattison  
Vincent Romano  
Paul L. Dubin  
Barry B. Muhoberac  
Department of Chemistry  
Indiana University Purdue  
University at Indianapolis  
Indianapolis, IN 46205

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## Complexation of Trypsin and Alcohol Dehydrogenase with Poly(diallyldimethylammonium Chloride)

*Complexation of alcohol dehydrogenase (ADH) and trypsin with poly(diallyldimethyl-ammonium chloride) (PDADMAC) in dilute electrolyte solution was studied by turbidimetric titration, quasi-elastic light scattering (QELS), and electrophoretic light scattering (ELS). Both QELS and turbidimetric titration show that PDADMAC forms complexes with ADH and trypsin in 0.01M NaCl solution at pH  $\geq$  6.8 and pH  $\geq$  9.2, respectively. These complexes take the form of stable coacervates in 0.01M, pH 11.0, phosphate buffer solution. QELS shows sizes of 400 and 315 nm for the coacervates of ADH-PDMDAAC and trypsin-PDMDAAC, respectively, while ELS reveals that these coacervates carry a net positive charge. Activity measurements show that both ADH and trypsin are enzymatically active in their coacervated states. Complexation of trypsin and PDADMAC was also studied by fluorescence in 0.01M, pH 11.0, phosphate buffer, and the protein emission was found to be quenched by complexation. The fluorescence quenching data show that trypsin retains its three-dimensional structure in the complex. These and other results are consistent with the quenching of the two tryptophans on the protein surface, but not the interior ones. © 1997 John Wiley & Sons, Inc.*

### INTRODUCTION

The study of protein–polyelectrolyte complexes has been an interesting subject<sup>1–15</sup> for more than four decades, in part because of the practical uses of complexation in protein separation. Early investigations of protein–polyelectrolyte complexes were carried out by Morawetz et al.<sup>10,11</sup> in the 1950s. These studies described the precipitation of liver catalase by various synthetic polyelectrolytes. Consequently, the purification of proteins by polyelectrolytes was proposed. So far, protein–polyelectrolyte precipitation has been successfully used to separate and isolate whey proteins, to fractionate egg white proteins, and to remove nucleic acids from Baker's yeast.<sup>12–15</sup>

In addition to protein separation, the use of the protein–polyelectrolyte complex as an enzyme carrier has been of interest, leading to studies of enzyme activity in complexes. For example, Morawetz and co-workers<sup>16</sup> studied the tryptic digestion of hemoglobin complexed with polyacrylic acid. It was found that polyelectrolyte activates the protein at extreme pH. Recently, Kokufuta reported a wide, active pH range for trypsin in trypsin–poly(vinyl alcohol sulphate) complexes.<sup>17</sup> Larionova and co-workers<sup>18</sup> found that some protein–polyelectrolyte complexes can provide thermal stability in preservation of biological activity. More recently, we found azide binding of hemoglobin molecules in complexes with polyelectrolytes (J. Xia, P. L. Dubin, and B. B. Muhoberac, unpublished).

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Proteins or enzymes are fairly sensitive to their environment. Very frequently, proteins are susceptible to denaturation by chemical or physical factors such as pH extremes, temperature, and the presence of organic materials. These drawbacks have frustrated the use of unstabilized enzymes in solution and have in many instances necessitated the use of large amounts of costly enzyme, i.e., substantially above those theoretically required. The use of any large amounts of catalyst is undesirable, as catalysts are generally expensive and some loss is expected for most processes. However, in the case of enzymes, the disadvantage of their use in large amounts is particularly acute not only because they are highly susceptible to denaturation but also because they are difficult to recover and remove. In this regard, efforts have been made to improve the recoverability of enzymes by immobilizing them on solid supports. However, this technique usually covalently modifies the enzyme and often results in its destabilization and loss of enzyme activity.

In this paper, we report studies on the separate complexation of trypsin and alcohol dehydrogenase (ADH) with poly(dimethyldiallylammonium chloride) (PDADMAC). We study the activity of these enzymes in the coacervate form of complexes. Coacervates represent a kind of aggregate colloidal particle in aqueous solution. These particles appear as floating microscopic droplets, which may fuse with each other but do not mix with solvent. Trypsin and ADH form a coacervate with PDADMAC in solutions at pH above their isoelectric points. These coacervates are quite stable, and easily separated by filtration. We consider that these coacervates may be better enzyme carriers than the immobilized systems, from the viewpoint of activity retention and ease of preparation.

## EXPERIMENTAL

### Materials

PDADMAC was a commercial sample of "Merquat 100" from Calgon Corporation (Pittsburgh, PA), possessing a nominal molecular weight (MW) of  $2 \times 10^5$ . Bovine trypsin was obtained from Sigma Chemical as 95–99% pure lyophilized protein, with isoelectric point (IEP) of 10.5. Horse liver ADH, with IEP = 6.8, was purchased from Boehringer Mannheim. All salts used in the present work were AR grade and obtained from Sigma. All solutions were prepared with deionized Milli-Q water.

## Methods

**Turbidimetry.** Turbidity measurements were made at 420 nm with a Brinkmann PC800 probe colorimeter, equipped with a 2.0 cm path-length fiber optics probe. "Type I" titrations were carried out at 24°C by adding 0.1 M NaOH, with a micrometer buret, to a protein–polyelectrolyte solution in 0.01 M NaCl, and recording 100% T. A protein blank correction was made under the same conditions in the absence of polyelectrolytes.

**Quasielastic Light Scattering (QELS).** QELS measurements were made at scattering angles from 30° to 150° with a Brookhaven (Holtville, NY) 72-channel BI-2030 AT digital correlator and using a Jodon (Ann Arbor, MI) 15 mW He-Ne laser. A 200 μm pinhole aperture was used for the EMI photomultiplier tube, and decahydronaphthalene (decalin) was used as the refractive index matching fluid to reduce stray light. We obtain the homodyne intensity–intensity correlation function  $G(q, t)$ , with  $q$ , the amplitude of the scattering vector, given by  $q = (4\pi n/\lambda)\sin(\theta/2)$ , where  $n$  is the refractive index of the medium,  $\lambda$  is the wavelength of the excitation light in a vacuum, and  $\theta$  is the scattering angle. For a Gaussian distribution of intensity profile of the scattered light,  $G(q, t)$  is related to the electric field correlation function  $g(q, t)$  by

$$G(q, t) = A[1 + bg(q, t)^2] \quad (1)$$

where  $A$  is the experimental baseline and  $b$  is a constant, which depends on the number of coherence areas that generates the signal ( $0 < b < 1$ ). The quality of the measurements was verified by ensuring that the difference between the measured value of  $A$  and the calculated one was less than 1%. In the present work, we analyze the autocorrelation functions by using the CONTIN program, which employs the constrained regularization method<sup>19</sup> to calculate the mean diffusion time,  $\langle\tau\rangle$ , which is related to the diffusion coefficient by

$$D = \frac{\lambda^2}{16\pi^2 \sin^2(\theta/2) \langle\tau\rangle} \quad (2)$$

More detailed discussions of QELS data analysis may be found in Refs. 20 and 21. From each  $D$  value, we obtain the Stokes' radius  $R_s$  by the Stokes–Einstein equation:

$$R_s = \frac{kT}{6\pi\eta D} \quad (3)$$

where  $k$  is Boltzmann's constant,  $T$  is the absolute temperature, and  $\eta$  is the viscosity of the solvent. The Stokes' diameter that we report ( $D_{app}$ ) is an apparent value, based on the assumption that  $\tau$  corresponds to translational diffusion alone; this assumption is supported by several previous investigations.<sup>22,23</sup>

## Electrophoretic Light Scattering (ELS)

ELS measurements were made at four scattering angles (8.7°, 17.4°, 26°, and 34.7°), using a Coulter (Hialeah, Florida) DELSA 440 apparatus. The light source was a 5 mW He-Ne laser ( $\lambda = 632.8$  nm). The total volume of sample chamber is about 1 mL. A rectangular channel runs through a 5 mm thickness of the insert, connecting the hemispherical cavities in each electrode. The electric field was applied at a constant current of 0.4 mA. The temperature of the thermostated chamber was maintained at 25°C. Electroosmotic corrections were determined by measuring the spatial flow profile in the chamber and taking the mobility readings at a distance 16% of the rectangular length from the respective walls of the chamber. This procedure was verified by using a DELSA electrophoretic mobility standard.

In ELS, the measured Doppler shift frequency  $\Delta\omega$  is given by

$$\Delta\omega = \frac{2\pi n}{\lambda} Eu \sin \theta \quad (4)$$

where  $E$  (volts/cm) and  $u$  [ $(\mu\text{m s}^{-1})/(\text{V cm}^{-1})$ ] are the applied electric field strength and electrophoretic mobility, respectively. Therefore,  $u$  can be directly obtained from the  $\Delta\omega$ . The  $u$  values obtained in this work are repeatable to within less than 10% error. Detailed discussion on ELS measurements can be found in several reviews.<sup>24–26</sup>

## Fluorescence and Absorbance Measurements

Fluorescence spectroscopy was performed on a Model 8000 spectrofluorometer (SLM Aminco, Urbana, IL). Excitation intensities were delivered through the grating monochromator at 0.5 nm band pass. Excitation and emission wavelengths were 280 and 350 nm, respectively. The sample (1.5 mL) was contained in a stirred, thermostated (25°C) quartz triangular cuvette. Spectral data were stored on the IBM PC. Emission intensity correction and integration were performed with the software supplied with the instrument.

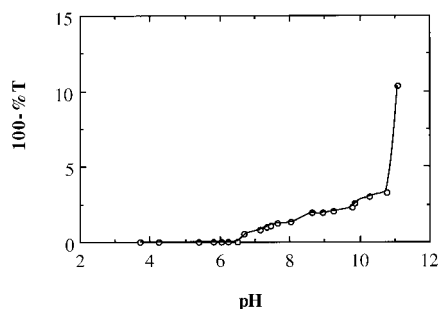
Absorbance measurements were performed on a Hewlett-Packard HP8450 spectrophotometer. The sample (1.5 mL) was contained in a thermostated (25°C) quartz 1-cm cuvette.

## Measurements of Enzyme Activity

ADH catalyzes the oxidation of alcohols to carbonyl compounds as exemplified in the following reaction:



By definition, one unit of ADH catalyzes the reduction of one micromole of  $\text{NAD}^+$  in 1 min at 25°C. Therefore,



**FIGURE 1** Type I turbidimetric titration of 0.10 g/L PDADMAC in 0.05 g/L ADH solution at ionic strength of 0.01 M NaCl.

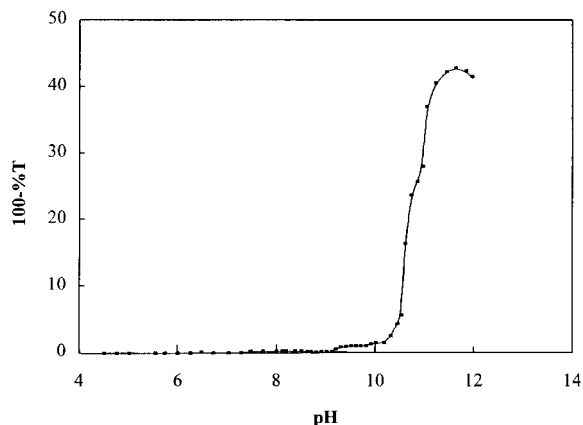
ADH activity can be determined from the velocity of the conversion of  $\text{NAD}^+$  to NADH. The reaction mixture contains 0.06 g/L of ADH, 2.0 mM  $\text{NAD}^+$  and 0.16 M  $\text{CH}_3\text{OH}$  in 0.01 M, pH 11.0, phosphate buffer. Complete mixing is assured by vortexing the solution for 20 s. After mixing, concentrations of NADH are measured at different time intervals by either absorbance at 340 nm or emission at 450 nm. To determine the ADH activity for ADH-PDADMAC complexes, the complex is first formed in 0.01 M, pH 11, phosphate solution, at concentrations of 0.06 g/L ADH and 0.12 g/L PDADMAC. The complex solution is then mixed with 2.0 mM  $\text{NAD}^+$  and 0.16 M  $\text{CH}_3\text{OH}$  by vortexing for 20 s in the same phosphate buffer solution. After vortexing, the solution is mixed with a magnetic stirrer, then sampled at different time intervals. Each aliquot is filtered into a quartz spectral cell through a 0.2  $\mu\text{m}$  filter. The solution obtained is diluted by adding an equal amount of phosphate buffer. After dilution, both absorbance and emission are measured.

Trypsin activity is obtained by following the catalytic hydrolysis of the substance N- $\alpha$ -benzoyl-DL-arginine-*p*-nitroanilide (BANA). Various amounts of BANA and 1.0 g/L trypsin (or trypsin-PDADMAC complex) are mixed in 0.01, pH 11, phosphate buffer, with the concentrations of BANA varying from 0.9 to 1.0 g/L. After mixing, the rate of BANA hydrolysis is determined by measuring the absorbance at 410 nm for formation of *p*-nitroaniline at different time intervals. Methods used for mixing and sampling are the same as described above for ADH.

## RESULTS AND DISCUSSION

### Complexation of ADH-PDADMAC, Trypsin-PDADMAC

Figure 1 shows a type I turbidimetric titration curve for 0.1 g/L PDADMAC in 0.05 g/L ADH solution at ionic strength ( $I$ ) of 0.01 M NaCl. The titration curve displays an abrupt increase in turbidity at pH



**FIGURE 2** Type I turbidimetric titration of 0.05 g/L PDADMAC in 0.25 g/L trypsin solution at ionic strength of 0.01 M NaCl.

11, corresponding to coacervation ( $\text{pH}_\phi$ ). Prior to reaching pH 11, a small turbidity increase is observed at pH 6.8 (the isoelectric point of ADH), corresponding to the initiation of complex formation ( $\text{pH}_c$ ). QELS measurements are carried out in solutions used for the type I titration, at both these transitions, i.e., pH 6.8 and 11.0. At pH 6.8, QELS shows  $D_s(\text{app}) = 140$  nm, which is larger than either pure ADH (7 nm) or PDMDAAC (48 nm) at the same conditions. At pH 11.0, QELS displays a much larger size, i.e., 400 nm. The 400 nm particle is quite stable; no changes are observed even after storing the solution at 4°C for a week. These results suggest that ADH and PDMDAAC start to form soluble complexes at pH 6.8. Upon increasing pH, complexes aggregate, and the size of complexes increases. Consequently, the turbidity increases. At pH 11, the aggregated complex forms a stable colloidal coacervate. Since the coacervate has a large diameter (400 nm), turbidity is quickly increased at this pH.

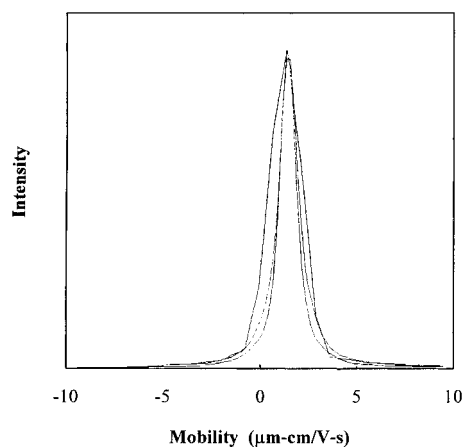
Figure 2 shows type I titration curve for trypsin–PDADMAC in 0.01 M NaCl solution. Concentrations of PDADMAC and trypsin are 0.05 and 0.25 g/L, respectively. In a manner similar to the titration for the ADH–PDADMAC system, a small turbidity increase is observed at pH 9.2, which is below the IEP of trypsin, prior to an abrupt increase of turbidity at pH 10.6. The corresponding apparent diameters for complexes formed at pH 9.2 and 10.6, respectively, are 75 and 140 nm. These diameters are much smaller than the comparable values obtained for ADH–PDADMAC complexes. There may be two factors causing the smaller diameters for trypsin–PDADMAC complexes. First, at  $\text{pH}_c$ ,

trypsin molecules are positively charged while ADH has zero net charge. The binding of trypsin at  $\text{pH} < \text{IEP}$  is likely to arise because of a negative charge patch,<sup>23</sup> but the positive proteins must cause considerable chain expansion of the host polycation. At  $\text{pH}_\phi$ , both trypsin and ADH molecules are negatively charged, but the net charge of the former is much smaller because  $\text{pH}_\phi$  is very close the IEP of trypsin. Second, the polymer:protein mass ratio ( $1/r$ ) is less than one for trypsin–PDADMAC but more than one for ADH–PDADMAC in the QELS measurements. Since polymer chains bridge complex particles, the larger size of ADH–PDADMAC complexes formed at higher relative polymer concentration is expected. For trypsin–PDADMAC at  $r < 1$  (0.2 and 0.1 g/L for the polymer and protein, respectively), QELS shows a very stable particle of 315 nm for the complex in 0.01 M, pH 11.0, phosphate buffer.

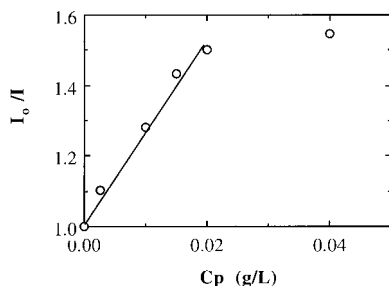
Phase transitions at  $\text{pH}_c$  and  $\text{pH}_\phi$  have also been studied for other protein–polyelectrolyte pairs.<sup>22,23</sup> These previous studies were mostly carried out at higher concentrations of both proteins and polymers. Different forms of phase separation may be observed at  $\text{pH}_\phi$ ; in the present case, we observe very stable coacervates beyond the phase separation point.

### ELS of ADH–PDADMAC and Trypsin–PDADMAC Coacervates

Figure 3 shows typical electrophoretic light scattering spectra for ADH–PDADMAC coacervate formed in pH 11.0 and 0.01 M phosphate buffer.



**FIGURE 3** Typical electrophoretic light scattering spectra for ADH–PDADMAC coacervates formed in 0.01 M phosphate buffer at pH 11.



**FIGURE 4** The Stern–Volmer plot for trypsin–PDADMAC complexation in 0.01 M phosphate buffer at pH 11.

Concentrations of ADH and PDMDAAC are 0.06 and 0.12 g/L, respectively. The average frequency of the spectrum gives a mobility  $u = 1.5 \mu\text{m-cm/V-s}$ . For trypsin–PDMDAAC coacervates, we obtain  $u = 1.7 \mu\text{m-cm/V-s}$  in the same phosphate buffer, with polymer and protein concentrations of 0.2 and 0.1 g/L, respectively. At the same conditions, the value of  $u$  for pure PDMDAAC is  $2.1 \mu\text{m-cm/V-s}$ . Values of electrophoretic mobility for coacervates are smaller than that for the pure polymer because positive charges on the polymer chain are partially neutralized by proteins in the coacervates.

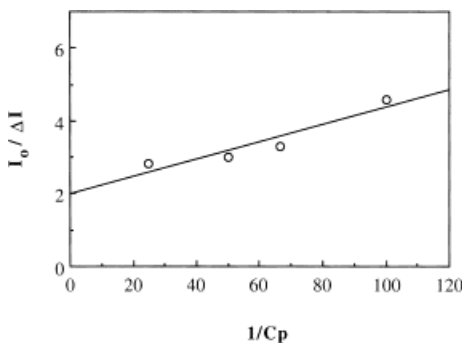
### Fluorescence Quenching Study of Trypsin–PDMDAAC Complexes

Quenching of the endogenous fluorescence has been used to study conformational changes of proteins,<sup>27–29</sup> most commonly with iodide and cesium ions as quenchers. Recently, we found that fluorescence quenching occurs with lysozyme–polyelectrolyte complexation.<sup>30</sup> In trypsin–PDMDAAC complexation, fluorescence quenching is also observed. Figure 4 shows a Stern–Volmer plot for trypsin–PDMDAAC complexation in pH 11.0, 0.01 M phosphate buffer, at a constant protein concentration of 0.2 g/L. Quenching of fluorescence intensity can be described by the Stern–Volmer equation:

$$I_0/I = 1 + K_q[Q] \quad (6)$$

where  $I_0$  and  $I$  are the fluorescence intensities in absence and presence of the quencher, respectively,  $[Q]$  is the concentration of quencher, and  $K_q$  is the Stern–Volmer constant. From the initial linear slope in Figure 4,  $K_q = 25.2 \text{ (L/g)}$  is obtained.

In order to investigate the possibility of a protein conformation change exposing the interior of the protein, the fluorescence intensities are replotted in



**FIGURE 5** Plot of fluorescence intensities in terms of the modified Stern–Volmer equation.

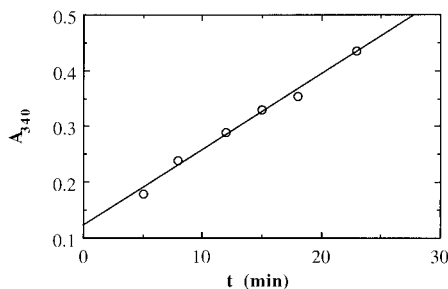
Figure 5 according to the modified Stern–Volmer equation:

$$\frac{I_0}{\Delta I} = \frac{1}{f_a K[Q]} + \frac{1}{f_a} \quad (7)$$

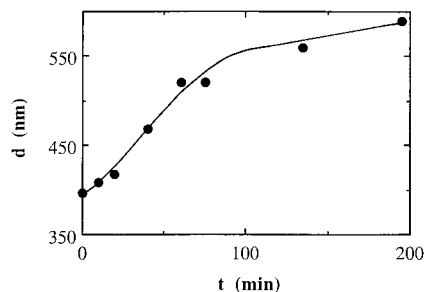
where  $\Delta I = I_0 - I$ , and  $f_a$  is the fraction of tryptophan molecules that are accessible to quencher.<sup>30</sup> Figure 5 shows  $f_a \approx 0.5$ , which suggests that about 50% of the tryptophan molecules in trypsin are quenched by PDADMAC by complexation. Two of the four tryptophan residues in trypsin reside on the protein surface (Polygen/Molecular Simulations, Inc., 200 Fifth Avenue, Waltham, MA 02154). Therefore, the present result is consistent with binding of PDADMAC at the protein surface without alteration of its native structure.

### Activity of ADH in ADH–PDADMAC Coacervates

Figure 6 shows the absorbance at 340 nm for solutions sampled at different times after mixing ADH–PDADMAC coacervates and  $\text{NAD}^+/\text{CH}_3\text{OH}$  substrates in 0.01 M, pH 11.0, phosphate buffer. From the slope of the linear plot in Figure 6, the enzyme



**FIGURE 6** The 340 nm absorbance as a function of time for mixtures of ADH–PDADMAC coacervates and  $\text{NAD}^+/\text{CH}_3\text{OH}$  substrates.



**FIGURE 7** Change of size with time for the ADH–PDADMAC coacervate during the enzymatic reaction.

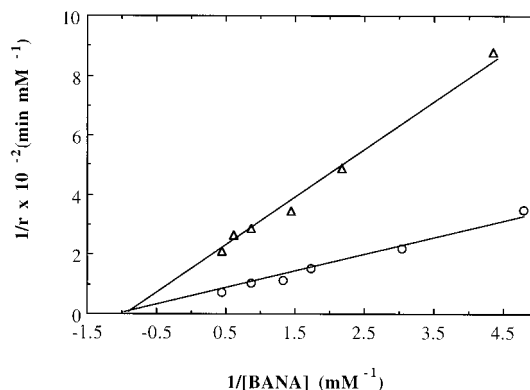
activity is calculated according to (Worthington Enzymes, Worthington Biochemical Corporation, 1992)

Units/mg protein

$$= \frac{\Delta A_{340}/\text{min}}{6.22 \times \text{mg protein/mL reaction mixture}} \quad (8)$$

An ADH activity of  $7.5 \times 10^{-2}$  units/mg is obtained for the ADH–PDADMAC coacervate in 0.01 M, pH 11.0, phosphate buffer. Under the same conditions, an activity of  $4.0 \times 10^{-2}$  units/mg is obtained for the pure protein, nearly two times less than that for the ADH–PDADMAC coacervate. PDADMAC may stabilize ADH upon complexation, protecting the enzyme from the alkaline environment. It is not yet known whether stabilization is the reason for the enhancement of activity, which could also arise from a shift of the pH-activity profile of the protein in the coacervate. The different local environmental around ADH in the coacervate can affect the activity of the protein assuming the protein is still bound to PDMDAAC after introducing  $\text{NAD}^+/\text{CH}_3\text{OH}$  into the coacervate solution. To address this point, we note from Figure 7 that the size of the ADH–PDADMAC complex increases with time during the enzymatic reaction. Since the size of the complex is quite stable in the absence of substrate (see above), this size increase appears to be related to the diffusion of substrate into the coacervate, and supports the proposal that catalysis occurs within the coacervate.

Since the size distribution of this system is not known in detail, it might be possible that QELS and activity measurements do not average over all species in an identical manner. Thus, the larger species that dominate the QELS result might not be the ones that predominate in the activity measurement. Nevertheless, we can state that the activity is not



**FIGURE 8** Lineweaver–Burke plots of trypsin and trypsin–PDADMAC coacervate in 0.01M phosphate buffer, at pH 11.

primarily due to uncomplexed protein, since we see an enhancement of enzymatic catalysis relative to that of free enzyme.

### Activity of Trypsin in Trypsin–PDMDAAC Coacervates

Figure 8 shows Lineweaver–Burke plots for trypsin and trypsin–PDMDAAC coacervates, respectively, in 0.01 M, pH 11, phosphate buffer. Concentrations of trypsin are 0.1 g/L in both of the cases, the polymer concentration is 0.2 g/L for trypsin–PDMDAAC coacervates. The hydrolysis rate ( $r$ ) of BANA is related to its concentration ( $[S]$ ) by the Michaelis–Menten equation:

$$\frac{1}{r} = \frac{K_m}{r_{\max}[S]} + \frac{1}{r_{\max}} \quad (9)$$

where  $K_m$  is the Michaelis–Menten constant and  $r_{\max}$  is the maximal hydrolysis rate. Values of  $K_m$  and  $r_{\max}$ , obtained from Figure 8 for both trypsin and the trypsin–PDMDAAC coacervate, are summarized in Table I. The identical  $K_m$  value for both

**Table I** Values of  $K_m$  and  $r_{\max}$  for both Trypsin and the Trypsin–PDADMAC Complex in 0.01M, pH 11.0, Phosphate Solutions

Samples	$K_m$ (mM)	$r_{\max}$ (mM min <sup>-1</sup> mg protein <sup>-1</sup> )
Trypsin	1.03	$3.34 \times 10^{-4}$
Trypsin–PDADMAC	0.95	$0.67 \times 10^{-4}$

trypsin and the trypsin-PDMDAAC coacervate suggests that the active site of the protein is not affected by the complexation of trypsin and PDMDAAC. On the other hand, the decrease of  $r_{\max}$  in the trypsin-PDMDAAC coacervate might be due to a shift of the pH-activity profile, as mentioned above.

In summary, we have studied coacervates of ADH-PDADMAC and trypsin-PDADMAC by turbidimetric titration, QELS, ELS, fluorescence, and enzymatic determinations. These coacervates have been shown to be physically stable and bioactive in 0.01 M phosphate solution at pH 11. Therefore, coacervates may be considered enzyme carriers for a wide range of applications.

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