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QUASI-ELASTIC LIGHT SCATTERING STUDIES ON PYRUVATE OXIDASE

T. RAJ [a,*] PATRICIA RUSSELL [a], W.H. FLYGARE [a] and ROBERT B. GENNIS [a,b,**]

[a] *Departments of Chemistry and* [b] *Biochemistry, University of Illinois, Urbana, Ill. 61801 (U.S.A.)*

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Summary

Quasi-elastic or dynamic light scattering has been used to examine the translational diffusion properties of the enzyme pyruvate oxidase (pyruvate:ferricytochrome b_1 oxidoreductase, EC 1.2.2.2.). Controlled proteolysis of the enzyme converts the native form of the enzyme to a protease-activated form which has a specific activity about 20-fold greater than the native oxidase. Light scattering studies indicate no significant change in the size or shape of pyruvate oxidase as a result of this proteolytic activation. In both cases the enzyme may be characterized as a hydrated sphere with a Stokes radius of about 53 Å. The sedimentation velocity-diffusion technique was used to obtain the molecular weight of this tetrameric enzyme, about 252 000 with a value of f/f_0 of 1.25.

Introduction

Pyruvate oxidase (pyruvate:ferricytochrome b_1 oxidoreductase, EC 1.2.2.2.) from *Escherichia coli* is a flavo-enzyme which catalyzes the oxidative decarboxylation of pyruvate to acetate plus CO_2 [1—4]. The native oxidase has been characterized as a tetramer consisting of four similar or identical subunits. The subunit molecular weight is about 60 000 [4]. This enzyme is a peripheral membrane enzyme which can be released from the bacterial membrane by sonication and purified to homogeneity. The specific activity of the pure enzyme can be stimulated more than 20-fold when assayed in the presence of a variety of lipids. Apparently this enzyme exists in a membrane-bound form with a high specific activity, and in a water-soluble form which has a low speci-

* Present address: Department of Chemistry, University of Minnesota, Minneapolis, Minn. 55455 U.S.A.

** To whom reprint requests and inquiries should be addressed.

fic activity. Alternatively the oxidase can be modified by trypsin to yield a protease-activated form of the enzyme which has the same specific activity and kinetic properties as the lipid-activated oxidase [5]. Pyruvate oxidase represents an excellent system for studying the role of lipids in activating membrane enzymes, and in studying the energetic interrelationships between protein-ligand, protein-protein and protein-lipid interactions. The study of the protease-activated form of pyruvate oxidase has been initiated in the hope of gaining insight into the nature of the membrane-bound or lipid-activated form of the enzyme.

In this paper the hydrodynamic properties of the native and protease-activated oxidase are compared in order to determine whether protease activation results in any large changes in the size and shape of the enzyme. Quasi-elastic light scattering was used to determine the translational diffusion coefficient of both forms of the oxidase. In addition, the sedimentation velocity-diffusion method was used to obtain a revised molecular weight of the native pyruvate oxidase.

Materials and Methods

Pyruvate oxidase. Pyruvate oxidase was prepared from *Escherichia coli* W-191-6 according to procedures previously described [4]. The final preparations were characterized by sodium dodecyl sulfate gel electrophoresis as being homogeneous. Enzyme activity was determined using the dichloroindophenol-reductase assay as previously described [2,4]. A ferricyanide-reductase assay was also employed. The pure enzyme preparations had specific activities ranging from 4500 to 6000 decarboxylase units per mg [1].

Trypsin activation. The conditions for trypsin activation of pure pyruvate oxidase are essentially the same as originally described by Hager for partially purified preparations [5]. The critical feature is that the presence of both the substrate (pyruvate) and cofactor (magnesium thiamin pyrophosphate) is required for protease activation. The specific activity of the oxidase was monitored after the addition of trypsin (Worthington) and when this reached the maximum value, soybean trypsin inhibitor (Miles) was added to stop the reaction. The ferricyanide-reductase assay was used to monitor protease activation.

Sample preparation. Due to problems relating to protein solubility, the native and protease-activated forms of pyruvate oxidase were examined under slightly different solution conditions. The native enzyme was examined in 0.1 M sodium phosphate, pH 5.7. This is the pH optimum for both enzyme activity and stability. The protease-activated oxidase was examined in a buffer consisting of the following: 0.1 M sodium phosphate, 0.1 M sodium pyruvate, 0.02 M magnesium chloride and 5 mM thiamin pyrophosphate at pH 5.7. All solutions were filtered three or four times through 0.22 μM Millipore filters which had been washed in the appropriate buffer. This was effective at removing dust particles and resulted in no loss of enzymatic activity. Protein concentration was generally in the range 0.2 to 1.2 mg/ml. For all measurements several different enzyme preparations were examined.

Light scattering. Details of the theory and instrumentation are available in several reviews of this technique [6—8]. These experiments were performed

using the homodyne (self-beat) method with an Ar^+ laser as a source of incident monochromatic light. The photocurrent was analyzed by a Federal Scientific UA-15A spectrum analyzer, and the output was averaged using a Federal Scientific 1015 averager. The resulting spectrum was fit to a single Lorentzian by a nonlinear least-squares procedure. In all cases the standard deviation was less than one percent. The half-width of this Lorentzian was used to compute the diffusion coefficient.

The physical basis of this technique is that laser light scattered from solutions of macromolecules is Doppler-broadened by Brownian motion. In the case of spherical particles, the spectrum of the scattered light is Lorentzian with half-width equal to DK^2 where D is the translational diffusion coefficient of the macromolecule and K is the magnitude of the scattering vector which is given by the formula $K = (4\pi n/\lambda_0)\sin(\theta/2)$ where n is the refractive index of the solution, λ_0 is the incident vacuum wavelength, and θ is the scattering angle. Measurements of the half-width at half-height of this spectrum lead to a direct determination of D. This technique is applicable to particles of virtually any size barring complications due to the effects of rotational diffusion. For these experiments all samples were maintained at $(20 \pm 0.1)^\circ$C. Homodyne spectra were collected for observed scattering angles ranging from 45° to 135°. The accuracy of the measurements was examined by measuring the homodyne spectrum for a sample of polystyrene latex spheres known to be 910 ± 60 Å in diameter (Dow Chemicals, Run No. LS-1132-B).

Sedimentation velocity. Sedimentation coefficients were obtained by measuring the sedimentation velocity using a Spinco Model E ultracentrifuge equipped with ultraviolet absorption optics. All measurements were made with a single-sector cell in an AN-D rotor at a rotor speed of 59 780 rev./min and a temperature of $(20 \pm 0.2)^\circ$C over a period of about 1 h. Photographs were usually taken about every 4 min. A Joyce-Loebl microdensitometer was used to obtain tracings of the photographs from which the boundary velocity was determined. The sedimentation coefficient was determined using standard procedures [9].

Values of both the diffusion coefficient and the sedimentation coefficient were determined at several different protein concentrations and extrapolated to infinite dilution. The molecular weight of pyruvate oxidase was calculated using the following equation [9]:

$$M_r = \frac{RTS^\circ_{20,w}}{D^\circ_{20,w}(1 - \rho_s \bar{v})}$$

where $R = 8.314 \cdot 10^7$ erg $\cdot$ mol^{-1} $\cdot$ degree^{-1} and $T = 293^\circ$K. The value of the solution density, ρ_s, was estimated to be 1.008 g/cm^3 [10]. The value of the partial specific volume of pyruvate oxidase, $\bar{v}$, has been previously determined by Williams and Hager to be 0.75 cm^3/g [1]. All values of the sedimentation coefficient and diffusion coefficient were corrected for both solvent viscosity and density according to standard procedures. Relative viscosity measurements were made using an Ostwald viscometer. The sedimentation constant of protease-activated pyruvate oxidase was not determined. The large amount of thiamin pyrophosphate required for protease activation made this solution

optically dense in the ultraviolet and hence necessitated the use of significantly higher protein concentrations. Attempts to remove the thiamin pyrophosphate led to difficult solubility problems of the protease-activated pyruvate oxidase. Conversely, there were considerable solution and stability problems encountered in attempting to study the native enzyme in the presence of pyruvate and thiamin pyrophosphate.

Results

Light scattering. A typical light scattering spectrum taken at 90° is shown in Fig. 1. Also shown in this figure is the best theoretical curve used to fit this data assuming a single Lorentzian. The angular dependence of the light scattering spectrum for both polystyrene latex spheres and native pyruvate oxidase solutions is shown in Fig. 2. The half-width at half-height is plotted against the square of the scattering vector. It can be seen that in both cases the predicted linearity is obtained. The diffusion coefficient for the polystyrene latex spheres determined in this manner is $4.68 \pm 0.04 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}. This compares with the value of $4.72 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1} calculated for spherical particles of this size using the Stokes-Einstein equation [9]. In Fig. 3B the concentration dependence of the diffusion coefficient of pyruvate oxidase is shown. There is a small negative dependence of $D_{20,w}$ with increasing protein concentration. The value of the diffusion coefficient extrapolated to infinite dilution is $4.05 \pm 0.1 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}. This corresponds to a Stokes radius of about 53 Å.

A typical time course for the trypsin activation of purified pyruvate oxidase

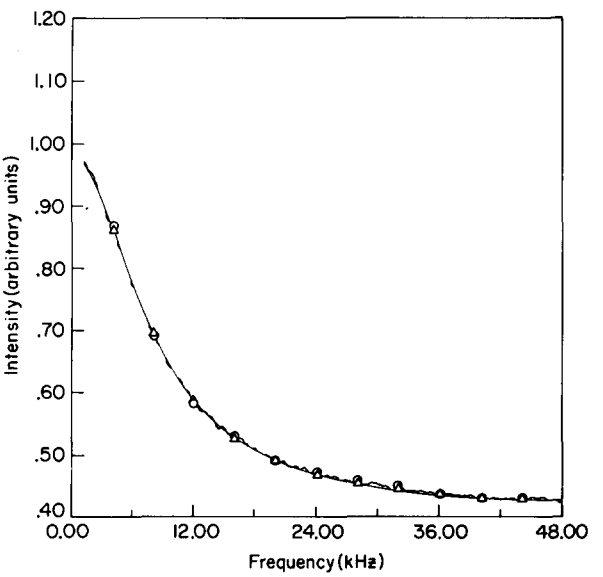

Fig. 1. Spectrum of the photocurrent for light scattered at $\theta = 90.0°$ by 1.13 mg/ml native pyruvate oxidase in 0.1 M sodium phosphate buffer, pH 5.7. Data was averaged for about 20 min. The experimental half-width at half-height is 6.48 KHz. The smooth curve (△———△) represents a calculated Lorentzian and the rough curve (○———○) is the experimental curve.

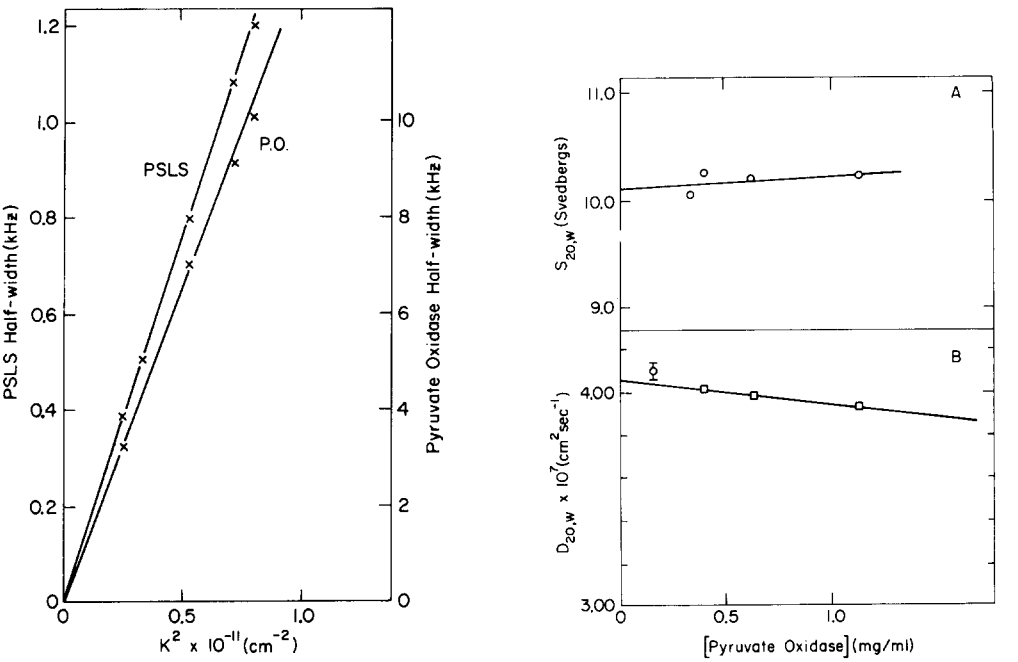

Fig. 2. Dependence of the observed half-width at half-height on K^2 for native pyruvate oxidase (P.O.) and polystyrene latex spheres (PSLS). Each point represents the average of at least three determinations.

Fig. 3. (A) Concentration dependence of the sedimentation coefficient of native pyruvate oxidase in 0.1 M sodium phosphate buffer, pH 5.7. (B) Concentration dependence of the diffusion coefficient of native pyruvate oxidase in 0.1 M sodium phosphate buffer, pH 5.7. The diffusion coefficient of protease-activated pyruvate oxidase at one concentration is also indicated. The error bar shows the deviation for measurements made on four different preparations.

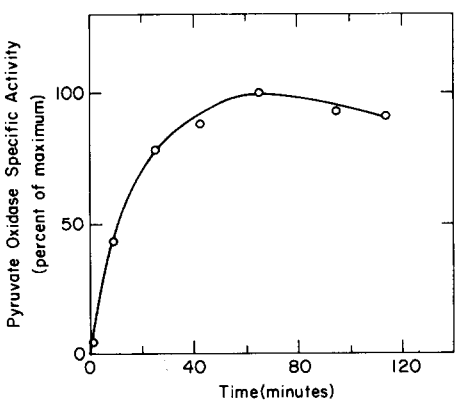

Fig. 4. Time course of the trypsin activation of pyruvate oxidase at room temperature. At t = 0, trypsin was added to start the reaction and at the indicated times aliquots were removed and assayed using the ferricyanide-reductase assay. The final mixture contained the following: 0.1 M sodium phosphate, 0.1 M sodium pyruvate, 5 mM thiamin pyrophosphate, 20 mM magnesium chloride, 0.5 mg/ml pyruvate oxidase and 10 μg/ml trypsin.

TABLE I

SUMMARY OF THE HYDRODYNAMIC PROPERTIES OF PYRUVATE OXIDASE

Native enzyme	$s^0_{20,w}$	10.1 S
	$D^0_{20,w}$	$4.05 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}
	f/f_0	1.25
	M_r	252 000
Protease-activated enzyme	$D_{20,w}$	$4.1 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}

is illustrated in Fig. 4. After about one hour under these conditions the specific activity of the enzyme increased about 20-fold. The diffusion coefficient was determined for several different enzyme preparations treated in this way. The diffusion coefficient of the protease-activated oxidase was virtually identical to that of the native enzyme within the uncertainty of the measurements. The average value of $D_{20,w}$ for four different preparations is $4.1 \pm 0.04 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}. This is also shown in Fig. 3B. The angular dependence of the light scattering again is consistent with a single, compact scattering particle (not shown).

Sedimentation of velocity. The sedimentation coefficient of native pyruvate oxidase was determined at several concentrations of protein. The results are shown in Fig. 3A. With increasing concentrations of pyruvate oxidase, there is a slight increase in the sedimentation coefficient. The value of $s_{20,w}$ extrapolated to infinite dilution is 10.1 S. This compares with the earlier value reported by Williams and Hager of 11.5 S. Using these values of $s^0_{20,w}$ and $D^0_{20,w}$ the molecular weight of native pyruvate oxidase was calculated to be 252 000. With this value for the molecular weight the value of f/f_0 was calculated where f_0 is the frictional coefficient for translational motion assuming the protein to be an anhydrous sphere. This ratio of frictional coefficients for native pyruvate oxidase is 1.25. The hydrodynamic data for pyruvate oxidase are summarized in Table I.

Discussion

The major motivation for this work was to compare the hydrodynamic properties of pyruvate oxidase prior to and subsequent to protease activation. This enzyme has the very unusual property that under certain conditions its specific activity is increased by more than 20-fold due to a proteolytic modification by trypsin. Several other proteases can also activate pyruvate oxidase under the same conditions [11 *]. The original observation of this phenomenon was made using an impure enzyme preparation. Subsequent work by Sweeney [12] and Poludniak [11] indicated a possible expansion or swelling of the enzyme after protease activation. It was felt that this would be a research problem to which quasi-elastic light scattering could be applied. The method is rapid, non-denaturing, and does not require large amounts of material for a protein of this size. No evidence of expansion or swelling of the enzyme was

* Also Russell, P. and Gennis, R.B., unpublished.

found. In the course of this work the hydrodynamic properties of native pyruvate oxidase were reexamined and extended beyond the previously reported work.

Earlier work by Williams and Hager [1] determined that the native enzyme molecular weight was 285 000 as determined by the Archibald method of sedimentation equilibrium. On the basis of the flavin (FAD) content the value of 266 000 was obtained for the molecular weight of the native tetramer. The value of the molecular weight using the sedimentation velocity-diffusion technique employed in this work is consistent with this value and with the subunit molecular weight recently estimated to be 60 000 to 65 000 [4]. The reason for the somewhat higher values of both the molecular weight (sedimentation equilibrium) and sedimentation coefficient previously reported [1] is not clear. However, it should be noted that there is a negative dependence of the diffusion constant and a positive dependence of the sedimentation coefficient with increasing oxidase concentration in the concentration range employed for this work (Fig. 3). This may indicate a tendency for the native enzyme to aggregate at the higher concentrations (2 to 20 mg/ml) used in the previous work. Houghton and Swoboda [15], in a short note, reported that the sedimentation of native pyruvate oxidase was reasonably independent of protein concentration between 1—5 mg/ml. The molecular weight was estimated to be 236 000. Experimental details were not given. It appears that buffer conditions, in particular, the presence of the enzyme cofactor and substrate can influence the tendency of this membrane enzyme to aggregate in solution.

The angular dependence of the light scattering (Fig. 2) and the value of f/f_0 of 1.25 is consistent with the hydrodynamic model of pyruvate oxidase as a hydrated sphere. These data do not, however, distinguish between the possible symmetries of this tetrameric enzyme, i.e., square planar or tetragonal. This is a question of particular interest for pyruvate oxidase because this enzyme is thought to interact with and bind to the surface of lipid bilayers and membranes, but it is not known how many of the subunits are involved in this interaction.

The light scattering data indicate that under the experimental conditions employed, there are no significant differences in the translational diffusion properties of the native and the protease-activated forms of the enzyme. The state of protein association for both unactivated and activated pyruvate oxidase under buffer conditions and at the low protein concentrations used in the enzyme assay (5—50 μg/ml) is currently being investigated. The data in this report make it appear probable that the activation of this enzyme by either lipids or by proteolysis does not involve changes in quaternary structure, i.e., dissociation or aggregation of pyruvate oxidase. This work also implies that no large peptides are being removed from the enzyme upon protease activation. This is consistent with the observation [11 *] that the subunit molecular weight of pyruvate oxidase is only reduced by approximately 10% upon protease activation (60 000 to 55 000). The light scattering data, however, does not clearly distinguish whether this small peptide, estimated to be about 5000

* See footnote p. 47.

daltons, is removed from each subunit. A reduction in the size of pyruvate oxidase by about 10% would result in an increase in the diffusion coefficient by only about 3%. This is just slightly larger than the uncertainty in these measurements. It does seem likely from these data, however, that the clipped portions of pyruvate oxidase do not dissociate from the main body of the enzyme upon proteolysis.

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