The Effect of Succinate on the Translational Diffusion Coefficient of Aspartate Transcarbamylase†

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ABSTRACT: Employing a differential optical mixing spectrometer, we have determined that the translational diffusion coefficient (D_T) of aspartate transcarbamylase (ATCase) decreases by (4.1 ± 0.6)% in the presence of succinate and carbamyl phosphate. This result, combined with the change in the sedimentation coefficient determined by Gerhart and Schachman (1968) and repeated by us in the present work indicates that ATCase experiences an increase in frictional coefficient of approximately 4% due to succinate and carbamyl phosphate, and that any change in the enzyme's partial specific volume ($\bar{v}$) under these conditions is less than about 0.3%. We have also measured $(D_T)_{20,w}$ for ATCase as $(3.75 \pm 0.11) \times 10^{-7}$ cm^2/sec. Combining this with our measured value of $s_{20,w}{}^0$ for ATCase of $(11.7 \pm 0.2) \times 10^{-13}$ sec and the calculated value of $\bar{v}$ of 0.738 cm^3/g (Rosenbusch and Weber, 1971), we have determined the molecular weight of ATCase as $(2.9 \pm 0.1) \times 10^5$. We have also observed the ATCase dimer and find that at a dimer concentration of 0.6 mg/ml the value of $s_{20,w}$ for the dimer is 15.8×10^{-13} sec and that this value decreases by (4.0 ± 0.5)% upon the addition of succinate and carbamyl phosphate, a behavior essentially identical with that of the monomer.

Aspartate transcarbamylase (ATCase) experiences an approximately 4% decrease in sedimentation coefficient (s) in the presence of succinate and carbamyl phosphate. This work (Gerhart and Schachman, 1968) has been interpreted to indicate that the enzyme undergoes a conformational change leading to an increase in frictional coefficient of 4%. This conclusion hinges upon the assumption that the partial specific volume ($\bar{v}$) of the molecule experiences an insignificant change under these circumstances.

In this paper we present a measurement of the change in the translational diffusion coefficient (D_T) experienced by ATCase in the presence of succinate and carbamyl phosphate, made using a differential optical mixing laser spectrometer to be described elsewhere (D. S. Cannell and S. B. Dubin, submitted to *Rev. Sci. Instrum.*). Because the diffusion coefficient depends solely upon the translational frictional coefficient (f_T) and is completely independent of the partial specific volume, combination of the value of the change in D_T with the previously measured value for the change in s leads to an unambiguous interpretation of the contributions of changes in $\bar{v}$ and f_T to the change in s. We find that the change in s experienced by ATCase in the presence of succinate and carbamyl phosphate is due entirely (within experimental error) to an increase in the enzyme's frictional coefficient, as has been previously suggested.

In the Methods and Results section of this paper we provide a brief description of the differential spectrometer and describe the following sequence of measurements and present the results obtained therefrom. The first measurement discussed is a repetition of the work of Gerhart and Schachman (1968), determining the change in sedimentation coefficient caused by the presence of succinate and carbamyl phosphate. We undertook this study to be certain that our sample preparation and handling techniques were adequate. In the course of these measurements we were able to study the behavior of the dimer of ATCase as well as that of the monomer, and to determine the relative concentration of the dimer. The second measurement presented is a determination of the translational diffusion coefficients of both the monomer and dimer of ATCase from the spectrum of the light scattered by a solution containing both forms. The unambiguous interpretation of this spectrum depends on knowledge of the dimer concentration and sedimentation coefficient obtained from the first measurement. The values of s and D_T found from these measurements are then used to obtain the molecular weight. The third measurement presented is that of the change in translational diffusion coefficient experienced by ATCase in the presence of succinate and carbamyl phosphate. Complete interpretation of this result also depends on knowledge of the behavior of the dimer.

Materials

The ATCase employed in these studies was kindly provided by Professor Glen Nagel. It was prepared according to the method of Gerhart and Holoubek (1967). Succinic acid (Baker) and glutaric acid (Baker) were recrystallized from benzene and ether, respectively. Dilithium carbamyl phosphate (Sigma) was purified in the manner of Gerhart and Pardee (1962). Stock solutions of dilithium carbamyl phosphate were stored frozen and thawed shortly before use. The standard buffer solution employed throughout was 40 mM potassium phosphate, 0.2 mM EDTA, and 2 mM mercaptoethanol, with the pH adjusted to 7.0.

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Methods and Results

I. Sedimentation Velocity Measurements. Sedimentation velocity measurements were made at 60,000 rpm with a Beckman Model E analytical ultracentrifuge. Schlieren optics were employed and the resulting photographs were analyzed on a Nikon microcomparator. By measuring two samples simultaneously in paired cells, one of which contained a wedge window (Gerhart and Schachman, 1968) we obtained both sedimentation velocity and difference sedimentation velocity data.

ATCase solutions were prepared in standard buffer solutions (see Materials section) which additionally contained either (i) 2 mM succinic acid and 2 mM dilithium carbamyl phosphate or (ii) 2 mM glutaric acid and an additional 2 mM potassium phosphate. Protein concentrations were determined spectrophotometrically ($A_{280}(1\%) = 5.9$) and sedimentation velocity data were obtained at ATCase concentrations of 1 and 5 mg/ml. At the higher concentration, a clearly defined dimer contribution was observed. From the relative areas of the dimer and monomer peaks on the schlieren plates, it was determined that 12% of the total enzyme present was in the dimer form. The resulting data for the monomer has been corrected to 20° and water, as well as to zero concentration employing the concentration dependence determined by Gerhart and Schachman (1968), and is displayed in Table I. It is noteworthy that both the dimer and monomer experience essentially the same decrease in sedimentation velocity in the presence of carbamyl phosphate and succinate. Both the values of s and Δs for the monomer are in good agreement with the work of Gerhart and Schachman (1968).

The sedimentation velocity data obtained at 1 mg/ml of enzyme concentration is also displayed in Table I. Once again, the values of the sedimentation velocity and its change in the presence of carbamyl phosphate and succinate are in agreement with that of the earlier work. However, the height of the dimer peak on the schlieren plates was too small at this concentration to determine values for s or Δs for the dimer. Since it is known that there is no equilibrium relationship between the monomer and dimer[1] we may assume that the fraction of dimer present was the same at 1 mg/ml of enzyme concentration as it was at 5 mg/ml. Furthermore, since the concentration dependence of s is so slight for the monomer (Gerhart and Schachman, 1968) we further believe that s for the dimer at an enzyme concentration of 1 mg/ml is approximately the same as measured at the higher concentration. As we will discuss below, these assumptions for the dimer's concentration and sedimentation velocity at the low concentration are necessary for a complete interpretation of the light scattering data, all of which was taken at an ATCase concentration of 1 mg/ml.

II. Diffusion Coefficient Determination. As described in previous papers (Dubin *et al.*, 1967; Dubin, 1972) the translational diffusion coefficient (D_T) of a macromolecule in solution is readily determined by measuring the self-beat spectrum of the light scattered by the solution. In the event that only one species is present this spectrum consists of a single Lorentzian. In the present case, this is somewhat complicated by the presence of a dimer component representing 12% of the total ATCase present. However, since we have already determined the sedimentation coefficient for both the monomer and dimer of ATCase as 11.7 ± 0.2 and 15.8 ± 0.3 S, respectively, we may deduce the value of D_T for both monomer and dimer from the spectrum of the scattered light. If we assume both species have essentially the same partial specific volume, the measured values of the sedimentation coefficients and the known molecular weight ratio (2) then imply (from the Svedberg equation) that the value of D_T for the dimer is 68% of that for the monomer. Our question then is reduced to the following: what is the self-beat spectrum of the light scattered by a solution containing 88% (by weight) of a species with molecular weight M and diffusion coefficient D_T (*i.e.*, the ATCase monomer) and 12% of a species of molecular weight $2M$ and diffusion coefficient $0.68D_T$ (*i.e.*, the ATCase dimer)? It is readily shown (Dubin, 1970,1972) that such a spectrum is highly Lorentzian in profile, and possesses a spectral width 92% as wide as that produced by a solution of monomer alone. We thus determined from the self-beat spectrum of the light scattered by a 1-mg/ml solution of ATCase that $(D_T)_{20,w}$ is given by $(3.75 \pm 0.11) \times 10^{-7}$ cm^2/sec for the monomer component and $(2.55 \pm 0.08) \times 10^{-7}$ cm^2/sec for the dimer component. Although this interpretation hinges on several plausible assumptions, it should be noted that the effect of allowing for the presence of the dimer is to reduce the measured value of D_T for the monomer by only 8%.

We may now immediately determine the molecular weight of aspartate transcarbamylase from the values of $s_{20,w}^0 = (11.7 \pm 0.2) \times 10^{-13}$ sec and $(D_T)_{20,w} = (3.75 \pm 0.11) \times 10^{-7}$ cm^2/sec as determined in the present work, and the calculated value of the partial specific volume of ATCase as 0.738 cm^3/g (Rosenbusch and Weber, 1971). These values yield from the Svedberg equation a value of $M = (290 \pm 10) \times 10^3$. This determination of M by the technique of sedimentation-diffusion compares well with values of M ranging from 300,000 to 310,000 as measured by a variety of different techniques (Gerhart and Schachman, 1965; Rosenbusch and Weber, 1971).

Table I: Summary of Sedimentation Coefficient Measurements on Aspartate Transcarbamylase.

ATCase Concn (mg/ml)	Solvent	$s_{20,w}^0$ (monomer) (units of 10^{-13} sec)	$s_{20,w}$ (dimer) (units of 10^{-13} sec)
5	Standard buffer + 2 mM glutaric acid and 2 mM potassium phosphate	11.7 ± 0.2	15.8 ± 0.3
5	Standard buffer + 2 mM succinic acid and 2 mM dilithium carbamyl phosphate	Decreases by (3.9 ± 0.5)%	Decreases by (4.0 ± 0.5)%
1	Standard buffer + 2 mM glutaric acid and 2 mM potassium phosphate	11.7 ± 0.2	
1	Standard buffer + 2 mM succinic acid and 2 mM dilithium carbamyl phosphate	Decreases by (3.6 ± 0.5)%	

[1] Professor Glen Nagel, personal communication (1974).

Table II: Summary of Differential Laser Probe Data on Aspartate Transcarbamylase.

Step No.	Cell 1	Cell 2	Wider Spectrum	% Difference
1	1 mg/ml of ATCase in standard buffer	1 mg/ml of ATCase in standard buffer	1	0.15 ± 0.4
2	1 mg/ml of ATCase in standard buffer	1 mg/ml of ATCase in standard buffer + 2 mM glutaric acid and 2 mM potassium phosphate (particulates present)	1	1.7 ± 0.4
3	1 mg/ml of ATCase in standard buffer + 2 mM succinic acid and 2 mM dilithium carbamyl phosphate	1 mg/ml of ATCase in standard buffer + 2 mM glutaric acid and 2 mM potassium phosphate (particulates present)	2	2.4 ± 0.4
4	1 mg/ml of ATCase in standard buffer + 2 mM succinic acid and 2 mM dilithium carbamyl phosphate	1 mg/ml of ATCase in standard buffer	2	4.6 ± 0.4
5	1 mg/ml of ATCase in standard buffer + 2 mM succinic acid and 2 mM dilithium carbamyl phosphate	1 mg/ml of ATCase in standard buffer + 2 mM glutaric acid and 2 mM potassium phosphate	2	5.6 ± 0.4

III. Change in Diffusion Coefficient. Although one might expect that small changes in the translational diffusion coefficient could be measured directly using the light scattering technique, this has not proved to be the case. Drifts in electronic components and in the quantum efficiency of the photomultiplier tube used to detect the scattered light generally limit the attainable accuracy to 1%. We have been able to overcome this problem by using a differential technique to measure directly the difference in the spectra of the light scattered from similar solutions contained in two optically contacted scattering cells through which the laser beam passed. A chopping wheel was used to alternately collect light from either cell. The resulting photocurrent was spectrally analyzed and phase sensitive detection employed to yield the difference in the two spectra. By varying the focusing of the beam in the cells it was possible to adjust the two spectra so that they had nearly the same amplitude at zero frequency, and by varying the amount of light collected from each cell it was possible to match the spectral amplitudes at very high frequencies. The resulting difference spectrum can be shown to have maximum amplitude at a frequency very nearly equal to the mean line width of the two spectra, and furthermore this amplitude is a direct measure of the difference in the line widths of the two spectra. We used a single channel spectrum analyzer to measure this amplitude in order to study small changes in the diffusion coefficient of ATCase.

The primary experimental difficulty we encountered in this study was due to the tendency of ATCase to aggregate slightly when passed through a Millipore filter. Light scattering measurements of D_T are quite sensitive to aggregation and particulate contamination of the sample for the following reason. Light scattered by the large contaminant is quite narrow spectrally; however, this light "beats" with the light scattered from the sample and produces a component in the photocurrent having a spectral width half as great as that due to the "beating" of the light scattered by the sample with itself. This effect is commonly referred to as "heterodyning," and can also be caused by accidentally collecting stray elastically scattered light. The resulting photocurrent spectrum is quite accurately Lorentzian; however, it has a half-width which is less than would be obtained in the absence of contamination. In fact, approximately a 5% reduction in line width occurs when 5% of the collected light is due to particulate contamination (Dubin, 1970,1972). Thus, in attempting to measure the change in D_T caused by the presence of succinate and carbamyl phosphate, it was important to verify that the observed change could not be attributed to the introduction of contamination. In attempting to do so, the following sequence of measurements was made. A cell filling and flushing apparatus was constructed so that cells could be cleaned, flushed, and dried without opening them to air. An array of inert valves (Hamilton) was employed so that various solutions could be added to or removed from the cells at will, and all such solutions could be admitted through the same Millipore filter which was flushed and cleaned *in situ* between the steps. The two optically contacted scattering cells were cleaned by repeated flushing with distilled water. The water was admitted to the cells through a 0.22-μ Millipore filter, which was then blown dry with filtered nitrogen. A 1-mg/ml solution of ATCase in standard buffer was then admitted to both cells simultaneously through the same filter. This filtration was carried out slowly, yielding 6 cm^3 of solution over a period of approximately 5 min. The difference in the diffusion coefficients of these two identical solutions was then measured using the differential laser probe. A concentrated solution of glutaric acid and potassium phosphate in standard buffer was then added to one cell through the filter, the resulting concentration of glutaric acid, and the increase in concentration of potassium phosphate both being ~2 mM. The difference in the diffusion constants was again measured and the results of these two steps are presented in Table II. Examination with a microscope of the light scattered by the sample containing glutarate revealed the presence of particulate contamination. We surmise that this was due to dislodging aggregates of ATCase from the downstream side of the filter when admitting the glutarate, as this effect has not been observed since that time, provided that the filter is cleaned by flushing with standard buffer after being used to filter ATCase. The purpose of admitting the glutarate was to demonstrate that solutions could be added to the cells containing ATCase without introducing significant contamination, and although we had failed to do this completely, the cell containing glutarate still constituted an excellent reference by comparison with which a

change in diffusion constant caused by admitting succinate and carbamyl phosphate to the other cell could be measured. In this regard it should be noted that although the presence of particulates reduced the effective line width by ~1.6%, the deviation of the resulting spectrum from a Lorentzian shape was completely negligible (Dubin, 1970). We therefore flushed the filter with standard buffer and admitted a solution of succinic acid and carbamyl phosphate to the cell containing only ATCase in standard buffer. This brought the concentrations of both succinic acid and carbamyl phosphate to 2 mM. We then measured the difference in the diffusion coefficients of the two samples and the results are presented in line 3 of Table II. Examination with a microscope of the light scattered from the cell containing succinate revealed no indication of particulate contamination. We thus conclude that the presence of succinate and carbamyl phosphate caused the translational diffusion coefficient of ATCase to decrease by (4.1 ± 0.6)%. In order to verify that we could add a solution to one of the cells containing ATCase, without artificially decreasing the spectral line width of the light scattered from the ATCase by the introduction of aggregates or particulate contamination, we emptied the cell containing glutarate by flushing it with standard buffer and blowing it dry with filtered nitrogen. We then filled this cell with ATCase in standard buffer, again forcing this solution through the filter very slowly. Microscopic examination of the scattered light revealed no contamination and we then measured the difference in the spectral line widths of the light scattered from the two cells and found it to be (4.6 ± 0.4)%. We do not consider this measurement to constitute a second determination of the change in line width caused by the presence of succinate and carbamyl phosphate since no comparison of the line width for the newly filled cell with the original line width of the cell containing succinate was possible. We next added a concentrated solution of glutaric acid and potassium phosphate in standard buffer to the refilled cell, again through the Millipore filter. This resulted in a 2 mM glutaric acid concentration and an additional 2 mM potassium phosphate concentration in that cell. We again measured the linewidth difference and found it to be (5.6 ± 0.4)%. The apparent 1% *increase* in line width upon addition of glutarate is nearly zero within the statistical errors with which we determined this change, and there is no reason to suppose that it is real. Any introduction of aggregates or particulate contamination would have caused a *decrease* in line width, and we therefore concluded that our techniques for adding solutions to the cells containing ATCase were adequate to prevent spurious changes in line width. The errors quoted on all of the line width difference measurements are one standard deviation in the measured line-width difference. These errors are a purely statistical effect due to the inherently random nature of the thermally induced diffusion of the molecules under study. They do not represent an inherent limitation of the accuracy of this differential technique and can be reduced substantially by using a multichannel device to simultaneously measure the spectral difference at many frequencies.

Conclusions

We have determined that aspartate transcarbamylase experiences a (4.1 ± 0.6)% decrease in translational diffusion coefficient in the presence of succinate and carbamyl phosphate. This change would be anticipated from the difference sedimentation data of Gerhart and Schachman (1968) on the assumption that any change in the enzyme's partial specific volume is negligible under these conditions, and hence our results corroborate this view.

Acknowledgments

The authors would like to express their sincere appreciation to Professor Glen Nagel for providing the samples of ATCase employed in these studies, as well as for many valuable discussions during the course of the work. We would also like to thank Ann Sanders for her help in taking the data.

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