Protein Aggregation

THE EFFECT OF DEUTERIUM OXIDE ON LARGE PROTEIN AGGREGATES OF C-PHYCOCYANIN

BY J. J. LEE AND D. S. BERNIS

*Division of Laboratories and Research, New York State Department of Health,
and the Department of Biochemistry, Albany Medical College of Union University,
Albany, N.Y. 12201, U.S.A.*

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The amount of 11s aggregate in phycocyanin, normally stimulated by hydrophobic forces, is dramatically increased by the presence of deuterium oxide. Proteins in which hydrophobic forces are not proposed as a mechanism for aggregation are unaffected by deuterium oxide. These observations are consistent with the lower critical micelle concentration reported for ionic detergents in deuterium oxide. Phycocyanin samples containing a majority of material sedimenting faster than 11s were also investigated in the presence of deuterium oxide with the following findings: the most rapidly sedimenting species in water buffer is 24s; in deuterium oxide more than 10% of the protein sediments at 67s and substantial amounts of other species with sedimentation coefficients larger than 24s are present. These large quantities of species sedimenting faster than 24s are found in deuterium oxide buffers from pD 5.5 to 7.0. Sucrose-density-gradient studies in deuterium oxide at pD 6.0 confirm the presence of large amounts of more rapidly sedimenting species. Spectrophotometric studies on fractions from the sucrose-density-gradient experiments indicate with the presence of higher aggregates a red shift of the visible-absorption maximum and an enhancement of the E_{620}/E_{280} ratio. Fluorescence-emission studies show a greater relative fluorescence efficiency for these higher aggregates and are consistent with the suggested enhancement of higher aggregates in deuterium oxide. The existence of phycocyanin aggregates of such a large size is suggested to be of importance *in vivo*, with phycocyanin playing a role as a structural protein.

The effect of deuterium oxide on protein aggregation has been investigated with a number of different proteins (Bernis, Lee & Scott, 1968; Paglini & Lauffer, 1968) and in at least one system *in vivo* (Inoué, Sato, Kane & Stephens, 1965). In studies from this Laboratory the greatest attention was given to the C-phycocyanin system. It was demonstrated that the amount of 11s hexamer was greatly enhanced in deuterium oxide. This observation is consistent with the suggestion that an important factor in stabilizing the 11s species is hydrophobic interactions. The critical micelle concentration of ionic detergents in deuterium oxide has been reported to be smaller than that found in water (Kresheck, Schneider & Scheraga, 1965; Emerson & Holtzer, 1967). In deuterium oxide, where there is a greater amount of localized structure than in water, there should be a greater entropic effect and consequently an enhancement of hydrophobic interactions (Kresheck *et al.* 1965).

In recent work we have been able to purify

phycocyanin and retain a preponderance of very large aggregates (Lee & Bernis, 1968). The presence of the higher aggregates is apparently not an artifact of purification since they are found in large quantities in crude algal extracts. Electron microscopy furnishes evidence (Luftig & Haselkorn, 1967; Gantt & Conti, 1967) that very large phycocyanin or other biliprotein aggregates or arrays are of importance *in vivo*. The question then must arise as to whether further aggregation, which is at least in part hydrophobically stimulated, can be enhanced by exposure of the large phycocyanin aggregates to deuterium oxide. A detailed study of these aggregation effects in deuterium oxide is the subject of this paper.

EXPERIMENTAL

Phycocyanin was isolated and purified from the blue-green alga *Phormidium luridum* (Lee & Bernis, 1968). Sedimentation-velocity measurements were made with a

Spinco model E analytical ultracentrifuge. These experiments were performed at ambient temperature (approx. 23°) and all S values were corrected to $S_{20,w}$. No attempt was made to determine sedimentation coefficients at infinite dilution; only $S_{20,w}$ values at single protein concentrations are reported. Sucrose-density-gradient studies were made according to the method of Martin & Ames (1961) with a Spinco model L-2 ultracentrifuge. These experiments were performed at 4° and corrections were applied to determine sedimentation coefficients. The $S_{20,w}$ values for each set of experiments were determined by the method outlined by Martin & Ames (1961) and by comparison with protein of known $S_{20,w}$ values run simultaneously in the model L-2 ultracentrifuge. Corrections for the use of D₂O as solvent were applied in a fashion completely analogous to that for H₂O experiments at a particular temperature. Kirshenbaum's (1951) data for viscosity and density of D₂O as a function of temperature were used. Sucrose-D₂O-density-gradient data were obtained by assuming the additivity of any differences between H₂O and D₂O viscosity and density data. Another assumption inherent in treating data from D₂O experiments so that $S_{20,w}$ values may be calculated is that, apart from a correction for deuteration of exchangeable hydrogen atoms, the partial specific volumes of the proteins do not vary from H₂O to D₂O. This treatment is consistent with the study by Edelstein & Schachman (1967). The correction suggested by these authors amounted to $k=1.0155$, where k is the ratio of the molecular weight of the protein in the deuterated to that in the non-deuterated solvent. The corrected $S_{20,w}$ values were therefore calculated by:

$$S_{20,w} = \left[S_{\text{obs.}} \left(\frac{\eta_{t,D_2O}}{\eta_{20,H_2O}} \right) \left(\frac{1 - \bar{v}\rho_{20,H_2O}}{1 - \frac{\bar{v}}{k}\rho_{t,D_2O}} \right) \right]$$

where the symbols have their usual meanings. All these assumptions may be valid to varying degrees; however, the composite effect is such that the $S_{20,w}$ values for such proteins as thyroglobulin and several phycoerythrins are quite closely reproduced in sucrose-D₂O density gradients, D₂O sedimentation-velocity experiments and H₂O experiments in the analytical ultracentrifuge. For the purposes of this investigation we report only $S_{20,w}$ values from sucrose-density-gradient experiments rounded to the closest integer, although certainly the precision is substantially better. All experiments were performed in sodium phosphate buffers, 1.0 M. Samples examined in the presence of D₂O were prepared by using 1 ml. of the protein solution in an appropriate H₂O buffer, freeze-drying the sample, reconstituting by addition of 1 ml. of D₂O and freeze-drying again. The sample reconstituted by adding 1 ml. of D₂O after the third freeze-drying was then examined. The pH of the sample was measured with a Radiometer TTT1a pH-meter and the pD was taken to be the observed pH + 0.4 (Glasoe & Long, 1960).

Spectra of all samples were measured with a Beckman DB spectrophotometer and a Cary model 14 spectrophotometer. Concentrated samples were measured by using a spacer cell so that the light-path was effectively 0.1 mm. Fractions from sucrose-density-gradient experiments were monitored with the Cary model 14 spectrophotometer and a Baird Atomic SF-1 spectrofluorimeter. Fluorescence measurements were performed as previously reported (Lee & Bernis, 1968).

Density-gradient experiments were also attempted with the analytical centrifuge with a technique similar to that of Vinograd, Bruner, Kent & Weigle (1963). In these experiments single-sector Kegeles-type (Kegeles, 1952) synthetic-boundary cells or the new Vinograd cells (Vinograd *et al.* 1963) for density-gradient centrifugation were used. Appropriate buffered solutions in D₂O were put into the body of the cell and buffered protein solution in H₂O was used in the small layering compartment. The results with these types of experiments were remarkably similar to those of the sucrose-D₂O-density-gradient experiments.

The relative amount of each species present in a centrifugation experiment is assumed to be proportional to the area under the schlieren peak, or the extinction peak in the sucrose-density-gradient experiments. In sedimentation-velocity experiments with the analytical centrifuge in the small concentration range investigated (5–25 mg./ml.) no change in areas as a function of concentration was encountered.

The fluorescence-excitation and -emission spectra of samples were measured with a Baird Atomic SF-1 spectrofluorimeter.

RESULTS

Several experiments were performed with phyco-cyanin purified at pH 7.0. These samples were exposed to appropriate deuterium oxide and water buffers and the relative amounts of species present were measured. These values are presented in Table 1 and are analogous to the more complete data reported by Bernis *et al.* (1968). They corroborate the initial findings of the effect of deuterium oxide on aggregation of phyco-cyanin, namely that

Table 1. *Typical results from water and deuterium oxide sedimentation-velocity experiments with phyco-cyanin*

H ₂ O			D ₂ O		
pH	$S_{20,w}$ (s)	Relative % of total area	pD	$S_{20,w}$ (s)	Relative % of total area
6.0	6.2	22	6.1	6.4	13
	10.3	55		10.8	69
	16.7	23		17.4	18
7.4	5.8	80	7.5	6.8	43
	10.4	16		11.6	41
	15.1	4		18.1	17
8.0	6.1	85	8.0	6.6	66
	11.1	11		11.4	29
	17.8	4		18.0	5

the amount of 11s species is enhanced in deuterium oxide at the expense of 7s aggregates and that the 19s species is apparently unaffected by the deuterium oxide. Sedimentation studies with other proteins in water and deuterium oxide were also performed. These proteins included those already reported (Berns *et al.* 1968), namely trypsin (bovine), soya-bean trypsin inhibitors, the trypsin-inhibitor 1:1 complex, catalase (bovine), α_s -casein B (bovine), phycoerythrin (*Porphyridium cruentum*) and thyroglobulin (pig). The results indicated no significant changes in observed $S_{20,w}$ values in

going from water to deuterium oxide for all proteins, except α_s -casein B, and no changes in relative amounts of areas under peaks where more than one species was present, such as in bovine catalase. Thyroglobulin appeared to have small changes in the relative amount of some higher aggregates.

Samples of phycocyanin purified at pH 6.0 were examined in deuterium oxide to investigate the effect of deuterium oxide on aggregates with sedimentation coefficient higher than 11s. These protein solutions at pH 6.0 had more than 50% of the material present as species sedimenting faster than 11s. In Table 2 the results for sedimentation-velocity experiments with the analytical centrifuge are presented. Most experiments were at a concentration of approx. 15mg./ml. Typical sedimentation patterns for water and deuterium oxide sedimentation-velocity experiments are shown in Figs. 1-4.

Table 2. *Effect of pH on sedimentation properties of large phycocyanin aggregates in water and deuterium oxide*

Results are for typical experiments at a single protein concentration of approx. 10mg./ml. and all samples were in sodium phosphate buffer, 1.0-1.

H ₂ O			D ₂ O		
pH	$S_{20,w}$ (s)	Relative % of total area	pD	$S_{20,w}$ (s)	Relative % of total area
5.5	5.6	16	5.5	5.9	10
	10.4	12		10.6	15
	16.0	14		16.9	10
	19.5	39		20.8	34
	23.8	19		30.4	20
6.0	5.3	27	6.0	5.3	15
	10.2	12		10.8	16
	16.0	13		16.7	11
	18.9	24		21.1	12
	22.2	24		28	33
6.5	5.4	19	6.5	5.2	13
	10.1	7		11.0	12
	15.2	8		17.1	9
	18.4	58		21.3	34
	23.4	8		30	31
7.0	5.5	20	7.0	5.1	16
	10.4	9		10.6	8
	17.0	72		16.6	7
				20.2	36
				27	20
8.0	5.2	60	7.5	5.5	30
	9.9	20		11.5	13
	14.5	20		17.2	14
				21.0	16
				25.1	27

Fig. 1. Sedimentation-velocity experiment with phycocyanin. Sedimentation was at 59780 rev./min. in the model E ultracentrifuge at approx. 23°, in sodium phosphate buffer, 1.0-1. Sedimentation is from left to right; each picture is at 32 min. (a) pH 5.5; (b) pD 5.5.

Fig. 2. Sedimentation-velocity experiment with phycocyanin. Sedimentation was at 59780 rev./min. in the model E ultracentrifuge at approx. 23°, in sodium phosphate buffer, 1.0-1. Sedimentation is from left to right. (a) pH 6.0, 32 min.; (b) pD 6.0, 16 min.; (c) pD 6.0, 32 min.

Fig. 3. Sedimentation-velocity experiment with phycocyanin. Sedimentation was at 59780 rev./min. in the model E ultracentrifuge at approx. 23°, in sodium phosphate buffer, 1.0. Sedimentation is from left to right; each picture is at 32 min. (a) pH 6.5; (b) pH 6.5, freeze-dried three times; (c) pD 6.5.

Fig. 4. Sedimentation-velocity experiment with phycocyanin. Sedimentation was at 59780 rev./min. in the model E ultracentrifuge at approx. 23°, in sodium phosphate buffer, 1.0. Sedimentation is from left to right; each picture is at 32 min. (a) pH 6.5; (b) pD 7.0.

Sucrose-density-gradient experiments were performed in deuterium oxide buffer, pD 6 and 1.0, in 5–20% (w/v) sucrose gradients. The plot of E_{620} versus fraction number is shown in Fig. 5. The approximate percentages of species found at each S value are listed in Table 3.

The fluorescence-excitation and -emission spectra of the several different sedimenting fractions indicated specific differences (Table 4). The most rapidly sedimenting species exhibited an excitation spectrum with a major peak close to 625 m μ with a shoulder evident at lower wavelengths. At later fractions, and consequently more slowly sedimenting fractions, the excitation peak at approx. 625 m μ broadened and the short-wavelength shoulder was more evident (Fig. 6). The most slowly sedimenting species showed the further trend of enhancement of

Table 3. Typical sucrose-density-gradient experiment with phycocyanin

The protein sample layered on the gradient was approx. 15 mg./ml. in 0.2 ml. of sodium phosphate buffer in D₂O, pD 6.0 and 1.0, and was centrifuged at 39000 rev./min. in the SW39 rotor for 6.5 hr. The extinction ratio is always reported at the visible-absorption maximum, which is approx. 620 m μ . It should be borne in mind that when shifts in the visible-absorption band take place, although this ratio is indicated as E_{620}/E_{280} , the extinction at the wavelength of the measured absorption maximum is shown.

$S_{20,w}$ (s)	Relative % of component	$\lambda_{max.}$ of visible peak (m μ)	E_{620}/E_{280} ratio
9	9	620	0.2 → 2.9
15	18	625	3.3 → 4.5
21	12	622	4.7 → 5.1
25	53	623	5.6 → 4.9
35	8	625–626	4.3 → 0.1

Fig. 5. Plot of E_{620} as a function of tube number (fraction number) of a sucrose–D₂O-density-gradient experiment with phycocyanin in sodium phosphate buffer, pD 6.0 and 1.0; a 5–20% (w/v) sucrose gradient was used, and sedimentation was for 6.5 hr. in the SW39 rotor.

the short-wavelength excitation and also a broadening at longer wavelengths. The emission spectra of the various fractions also exhibited slight changes (Fig. 7). The $\lambda_{max.}$ for the fluorescence emission was at approx. 647 m μ for the heaviest species and shifted slightly down to 645 m μ ; however, as the longest-wavelength fluorescence-excitation species began to appear, the emission maximum began to shift to longer wavelengths and was 653 m μ for the slowest species. This phenomenon is undoubtedly associated with the appearance of the so-called 'allophycocyanin' fraction (Lee & Bernis, 1968).

Table 4. *Typical fluorescence studies of phycocyanin*

The phycocyanin sample, pD6-0 and I 0-1, was from the sucrose-density-gradient experiment in Fig. 5. The fraction number is the same as the tube number indicated in Fig. 5. The arbitrary relative fluorescence efficiency is calculated as being the ratio of the fluorescence-detector reading (in μA) at the wavelength of the fluorescence maximum to the extinction at the visible-absorption maximum in a 1 cm. cell. All spectrofluorimeter sensitivity settings were kept constant and the excitation wavelength was always that of the absorption maximum.

Fraction no.	Approx. $S_{20,w}$ (s)	λ_{max} of fluorescence emission ($m\mu$)	Relative fluorescence efficiency
Original sample		647	55
15	> 39	647	54
22	28	646	82
24	23	645	43
28	16	649	42
33	< 10	653	34

Fig. 6. Typical fluorescence-excitation spectra of phycocyanin fractions from a sucrose-density-gradient experiment (the same as in Fig. 5). The fluorescence at $650 m\mu$ is measured in arbitrary units as a function of exciting wavelength. The concentrations used for curves A and B are not equal. The fractions correspond to those in Fig. 5. Curve A (—), tube no. 15, >39s; curve B (---), tube no. 33, <10s.

DISCUSSION

The simplest interpretation of the observed changes encountered in deuterium oxide solutions is that, since the critical micelle concentration in deuterium oxide for at least some detergents has been demonstrated to be lower than that in water (Emerson & Holtzer, 1967; Kresheck *et al.* 1965), one would predict an intensification of hydrophobic interactions in deuterium oxide over that in water. In several previous studies it has been demonstrated

Fig. 7. Typical fluorescence-emission spectra of phycocyanin fractions from a sucrose-density-gradient experiment (same as in Figs. 5 and 6). —, Tube no. 17; ---, tube no. 33. Excitation is at the visible-absorption maximum, in this case 624 and $620 m\mu$ respectively.

that the stabilization of the 11s or hexamer aggregate is at least in part favoured by hydrophobic interactions (Scott & Berns, 1965, 1967; Berns & Scott, 1966), although it is suggested that, once hydrophobic forces push the sub-units together, the dispersion force becomes predominant in stabilizing the higher aggregates. The enhancement of the amount of 11s aggregate in the presence of deuterium oxide, reported by Berns *et al.* (1968) and confirmed in this study, is consistent with the suggestion that hydrophobic forces play a role in stabilization of the 11s aggregate. The investigation of deuterium oxide effects with other proteins that aggregate, but with which a hydrophobic mechanism has not been suggested, demonstrated that no change in aggregation behaviour was present. With α_s -casein B an enhancement in aggregation was encountered. Thyroglobulin when examined in deuterium oxide has all of the species found in water buffers, and in approximately the same relative amounts. There is, however, some indication that a small enhancement of larger aggregates may occur. The published data for α_s -casein B (Ho & Chen, 1967; Payens & Schmidt, 1965) definitely suggest some possible role for hydrophobic

forces in stabilizing higher aggregates. The possibility of the importance of hydrophobic forces in enhancing large aggregates of thyroglobulin has not been considered (Edelhoc, 1960; Edelhoc & de Crombrughe, 1966) and the effects in the current investigations are not sufficiently large to suggest a hydrophobic mechanism without further studies.

Once it had been demonstrated that phycocyanin with higher aggregates could be prepared in buffers, pH 6.0 and 1.0, the natural question was: would further aggregation occur in deuterium oxide solutions? The initial deuterium oxide studies (Bernis *et al.* 1968) indicated that, whereas the amount of 11s aggregate was increased in deuterium oxide, the amount of 19s aggregate was essentially unaffected. In studies of the effect of deuterium oxide on phycocyanin solutions that contain most material sedimenting faster than 11s, the amount of 19s material is never enhanced. There was in the presence of deuterium oxide a large increase in aggregates higher than 19s, namely 22s, 30s and 64s species. It seems evident that these aggregates may represent the hexameric arrays reported by Luftig & Haselkorn (1967) in phage-lysed Cyanophyta and biliprotein particles reported by Gantt & Conti (1967).

The question immediately arises as to whether these large aggregates are of importance *in vivo*. In crude extracts and purifications of the algae *Plectonema calothricoides* and *Phormidium luridum* the largest aggregates have been approx. 25s. However, we have been able partially to purify phycocyanin from *Anacystis nidulans* with as much as 80% of the protein sedimenting at approx. 35s (J. J. Lee & D. S. Bernis, unpublished work). It would consequently seem that the highest aggregates may indeed be more than artifacts. Other evidence also indicates that deuterium oxide *in vivo* has the effect of increasing aggregation. Inoué *et al.* (1965) have reported that spindle protein structures in mitosis *in vivo* increase very significantly at higher concentrations of deuterium oxide. This aggregating protein system also appears to have properties associated with hydrophobic forces. Preliminary electron-microscopic investigation of the phycocyanin aggregates in the presence of deuterium oxide indicates that a considerable number of hexamers conglomerate to form higher aggregates.

The present investigation presents good evidence that deuterium oxide is a solvent that enhances hydrophobic interactions. The use of deuterium

oxide is a convenient method for testing the relative importance of hydrophobic forces in stabilizing various protein aggregates. This work also appears to support the suggestion that phycocyanin acts as a structural type protein in Cyanophyta and Rhodophyta. The phycocyanin that can be as much as 25% of the total algal cell may possibly polymerize into a hexameric array in which other parts of the photosynthetic system may be imbedded. In this regard, phycocyanin might be viewed as a prototype system for studying structural protein assembly, perhaps even that of protein associated with membrane formation.

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