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Characterization and Physical Properties of the Major Form of Phospholipase A₂ from Cobra Venom (*Naja naja naja*) That Has a Molecular Weight of 11,000*

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The major form of phospholipase A₂ from cobra venom (*Naja naja naja*) was prepared in 30% yield and was homogeneous on polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate and on Sephadex G-100 chromatography. The monomer molecular weight is about 11,000 according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ultracentrifugation and molecular sieve techniques were employed to confirm the molecular weight and to demonstrate a concentration-dependent aggregation of the enzyme. It was found that at concentrations below about 0.05 mg ml⁻¹, the enzyme exists predominantly in the monomeric form; kinetic studies are usually conducted in much more dilute solutions (0.2 μg ml⁻¹). The amino acid composition of the enzyme is reported. Of special interest is the presence of five to six disulfide bonds, 1 tryptophan residue, and 1 histidine residue. It is stable at high temperatures and is unusually resistant to denaturing agents. The isoelectric point was found to be 4.95. The findings that the protein is unusually resistant to denaturing agents and that it undergoes a concentration-dependent aggregation help to explain some of the previous reports in the literature on the apparent multiple forms of the cobra enzyme and their separation.

Phospholipase A₂ (EC 3.1.1.4) is one of the smallest enzymes in lipid metabolism and as such holds a unique potential for elucidating the nature of enzymatic activity on lipid aggregates. The importance of phospholipase A₂ for such studies (1, 2, 3) coupled with its widespread use in solubilizing membrane-bound proteins (4, 5) and in studying membrane structure (6) has led to the purification of phospholipase A₂ from many sources (7). The enzyme obtained from the cobra is most attractive for mechanistic studies (8, 9) because it is the smallest, readily available phospholipase A₂.

The main difficulty in purifying the cobra phospholipase A₂ is the fact that as many as 14 different forms of the enzyme have been detected in a single sample of cobra venom (5, 10, 11). Multiple forms of this enzyme also occur in other venoms (2) and in the pancreas (12). Several studies on the multiple forms of the cobra enzyme have been carried out. The goal of these studies was to separate the enzyme forms and to compare their characteristics. Salach *et al.* (5) used electrofocusing, followed by a low yield, laborious removal of ampholines. Shiloah *et al.* (11) used a large number of ion exchange and Sephadex G-50 columns. Both procedures were tedious and apparently failed to separate completely the enzyme forms. Since we were interested in obtaining a single, pure, small molecular weight form of the enzyme for use in mechanistic studies, we were able to bypass the more complicated proce-

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dures mentioned above by modifying the purification procedure of Braganca *et al.* (13). This relatively simple procedure produces, in 30% yield, a homogeneous protein with a molecular weight of only 11,000. The purification and physical characterization of this phospholipase A₂ are given below and kinetic studies with this enzyme are presented in the accompanying paper (14).

EXPERIMENTAL PROCEDURE

Venom from the cobra *Naja naja naja* (Pakistan), Lot no. NNPOL, was obtained from the Miami Serpentarium in the form of a lyophilized powder. Egg phosphatidylcholine (Schwarz/Mann) and Triton X-100 (Rohm and Haas) were employed in enzymatic assays. The radiolabeled phospholipid used to demonstrate enzyme specificity was 1-palmitoyl,2-palmitoyl [9,10-³H]phosphatidylcholine (Applied Science Laboratories), which was diluted with dipalmitoyl phosphatidylcholine (Calbiochem). Acrylamide (Eastman), *N*-bromosuccinimide (Eastman), sodium dodecyl sulfate (Sigma), and urea (Sigma) were recrystallized before use. CM-cellulose (0.7 meq/g), medium mesh (Sigma), and Sephadex G-100 (40 to 120 μ) (Sigma) were prepared according to the manufacturer's instructions. All other chemicals were of reagent grade.

Enzymatic activity toward egg phosphatidylcholine in mixed micelles with Triton X-100 was followed by the pH-stat technique (8). One unit of activity is the amount of enzyme required to hydrolyze 1 μmol of phospholipid/min. Protein was determined by the method of Lowry *et al.* (15), except where the enzyme's absorbance was employed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the procedure of Weber and Osborn (16), except that the denaturing solution also contained 4 M urea and was incubated at 37° for 1 hour.

RESULTS AND DISCUSSION

Purification—Phospholipase A₂ was purified using a modification of the procedure of Braganca *et al.* (13). The results of a

typical purification are summarized in Table I. The lyophilized crude venom was dissolved in glass-distilled H₂O at a protein concentration of 20 mg ml⁻¹. Undissolved material was removed by centrifugation (8,000 × g, 4°, 10 min), and 4 volumes of 6% perchloric acid were added to the clear supernatant at 4° with stirring. After the protein was allowed to set for several hours, the precipitate was collected by centrifugation (8,000 × g, 4°, 30 min). The precipitate was dissolved in a minimum amount of H₂O, and the pH was adjusted to 8.0 with 1 N NaOH. Since a white precipitate existed between pH 5 and 9, the solution was centrifuged (8,000 × g, 4°, 10 min), and the precipitate was discarded. The loss of activity in this step varied from 5 to 20%, whereas the protein loss was 30 to 50%. In all cases, there was an increase in specific activity.

The clear supernatant was eluted in two batches from a CM-cellulose column (2.5 × 50 cm) which had been equilibrated with 5 mM sodium phosphate buffer, pH 7.5. The protein was eluted with a step gradient of 5, 100, and 500 mM phosphate buffers at pH 7.5. The major active peak came off close to the breakthrough volume of the column and only required the use of 5 mM phosphate for elution. Two other peaks eluted from the CM-cellulose column and contained between 10 and 20% of the original activity. The first CM-cellulose peak was lyophilized and taken up in H₂O to a volume 1/10 of that of the original volume. This protein was then eluted from a Sephadex G-100 column (2.5 × 50 cm) in two batches with 50 mM sodium phosphate buffer, pH 7.5. Two peaks were routinely obtained on this column. The first came in the void volume and contained less than 10% of the protein and only a trace of activity. The second peak contained the majority of the activity and protein. A smaller inactive peak, eluting after the major peak, was found in some preparations. It should be noted that in our experiments the heat treatment step used by Braganca *et al.* (13) did not yield an increase in specific activity; in fact, there was a slight loss of total activity. Therefore, the heat treatment step was not included in our purification procedure.

Purity—The purified enzyme, when re-chromatographed on Sephadex G-100, gave the chromatogram shown in Fig. 1. Protein (Lowry method) determinations indicate that the amount of void volume protein (Peak I) is negligible, but the absorbance (A_{285} or A_{280}) of that peak is significant relative to the main peak. Presumably, the void volume peak is not due to heterogeneity but rather represents a small amount of aggregated or denatured phospholipase A₂ whose light-scattering effects elevate the solution's optical density. It was found that the size of this void volume peak depended upon the length of

time that the pure enzyme solution had been stored and whether it had been lyophilized. In fact, upon setting (4°), concentrated solutions of the pure enzyme would turn opalescent. A 2-mg ml⁻¹ enzyme solution was stored in the refrigerator for 1 month. Removal of the precipitate by centrifugation left the clear supernatant with only 91% of the original activity and 91% of the original protein. An identical solution stored frozen for the same period of time remained clear and lost neither activity nor protein when centrifuged. Opalescence was also observed when lyophilized samples were redissolved. In all cases, the solutions could be cleared by centrifugation (10,000 × g, 4°, 15 min). Because of this result, all enzyme samples were stored frozen, and if lyophilization was necessary, the redissolved protein solutions were centrifuged to remove any precipitate.

The contention that this preparation is homogeneous and that the void volume peak is an extremely small amount of aggregated or denatured phospholipase A₂ is supported by the fact that this same enzyme preparation gave one band on polyacrylamide gel electrophoresis at pH 8.5. One band was also found on sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the experimental conditions described under "Experimental Procedure."

Molecular Weight and Enzyme Aggregation of Phospholipase A₂—Phospholipase A₂ when run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis against standard proteins gives a monomer molecular weight of about 11,000. Analytical ultracentrifugation was employed to verify this value of the molecular weight. The plot of $\log c$ versus r^2 obtained from equilibrium sedimentation experiments exhibited a positive curvature. This result could indicate that the protein was undergoing rapid association-dissociation or that it was poly- or pausidisperse. Since the Sephadex G-100 and polyacrylamide studies indicated that this preparation was homogeneous, it was assumed that this curvature was due to protein association.

This assumption was substantiated by sedimentation velocity experiments. Fig. 2 shows the effect of protein concentration on the sedimentation coefficient. This type of behavior is characteristic of protein association (17). Extrapolation to zero concentration gives a sedimentation coefficient of about 1.8 to 1.9 S. It should be noted that the lowest protein concentration employed, 0.05 mg ml⁻¹, approaches the lower limits of the photoelectric scanning system. In addition, the determination

TABLE I

Purification of phospholipase A₂ from *Naja naja naja* venom

Procedure	Activity	Protein	Specific activity	Yield	Purification
	units	mg	units mg ⁻¹	%	
Crude venom	329,817	8458	39.0	100	1.0
Supernatant crude venom	320,262	8112	39.5	97	1.0
Neutralized HClO ₄ ppt	237,639	1616	147	72	3.8
Supernatant after centrifugation	192,995	844	228	59	5.9
CM-cellulose	99,170	341	290	30	7.4
Sephadex G-100	92,349	172	537	28	14

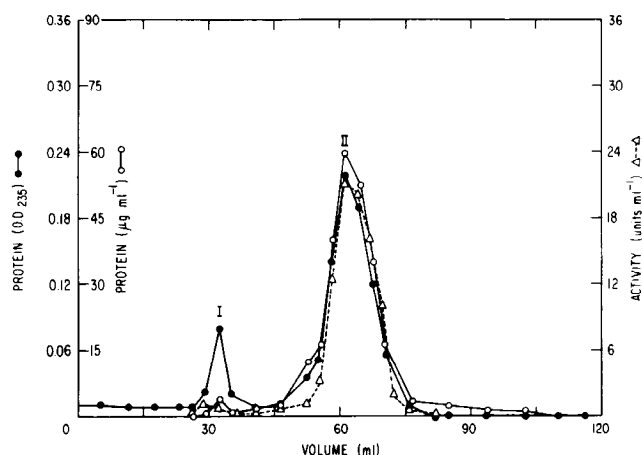


FIG. 1. Elution pattern of lyophilized phospholipase A₂ on Sephadex G-100 column (1.5 × 50 cm).

of sedimentation coefficients below 2 S are more sensitive to various systematic errors. Therefore, the extrapolation to zero concentration is subject to a large uncertainty. These complications are compounded by the difficulty of measuring diffusion coefficients under these conditions. Therefore, a calculation of the precise molecular weight of the monomer was not attempted. A very rough approximation can be obtained, however, by assuming the enzyme is an unhydrated perfect sphere with a sedimentation coefficient of 1.8 (18). This gives a minimum molecular weight of about 10,000. Thus Fig. 2 is consistent with a monomer-dimer and possible higher order association of an 11,000 molecular weight monomer.

To obtain an independent estimate of the monomer's molecular weight and as an additional confirmation of the enzyme's association, solutions with high and low protein concentrations were evaluated by Sephadex G-100 gel filtration. Protein concentrations were run so that the peak fraction eluting from the column had a protein concentration of less than 0.02 or greater than 1 mg ml⁻¹ (Fig. 3). The dilute sample gave an apparent molecular weight of 11,700 ± 1,000, whereas the more concentrated sample gave an apparent molecular weight of 20,000 ± 700. These molecular weights are based on the K_{av} values of three experiments.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, analytical ultracentrifugation, and Sephadex G-100 gel chromatography experiments are all consistent with a molecular weight for the monomeric phospholipase A₂ of about 11,000. In addition, the latter two experiments clearly show that the enzyme undergoes a concentration-dependent association between 0.05 and 0.5 mg ml⁻¹. The centrifuge experiments also indicate that higher order aggregates may exist above 2 mg ml⁻¹. In light of the remarkable stability of this enzyme, indicated by the fact that heating at 100° for 10 min results in only a minor loss of activity (13), it seems reasonable that the small amount of large molecular weight material found on Sephadex G-100 columns and the opalescence found after lyophilization were due to this concentration-dependent association rather than to denaturation. It should be noted that in the assay mixture employed here the enzyme concentration in

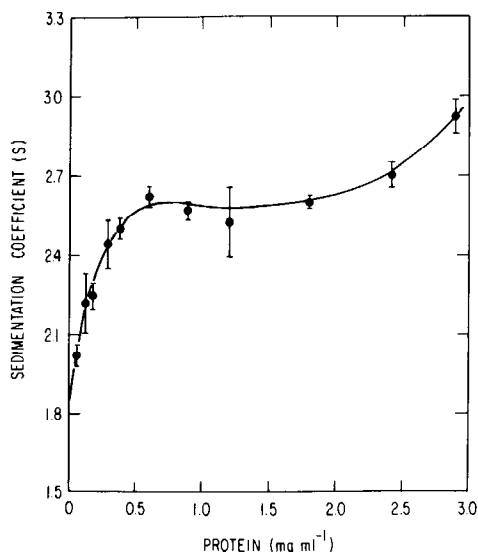


FIG. 2. Plot of apparent sedimentation coefficient versus initial enzyme concentration. The error bars indicate the standard deviation of at least three separate runs at each concentration. Runs were made at 20° and 59,780 rpm on a Beckman model E ultracentrifuge equipped with absorption optics and a photoelectric scanner.

the assay mixture is 0.2 μg ml⁻¹ which is 100 to 250 times less than the lowest concentration employed in the aggregation experiments. Thus, barring any large perturbations caused by the presence of Ca²⁺ or detergent-phospholipid mixed micelles, it would appear that under these assay conditions the enzyme exists in the monomeric form.

Isoelectric Point and Multiple Forms of Phospholipase A₂—Fig. 4 shows a typical electrofocusing experiment. There was one main peak (Peak II) and a second smaller peak (Peak I). Peak I represented only a very small portion of the total activity. The isoelectric point of the main peak was 4.95 to 4.97, whereas that of the smaller peak was 4.6 to 4.7. Since electrofocusing creates sharp protein concentration gradients and since the enzyme undergoes a concentration-dependent aggregation, these peaks could be due to an equilibrium between two aggregated forms of the enzyme. A second possibility is that Peak I, which contains a low level of activity, is the same high order aggregated form of the enzyme that was detected on the Sephadex G-100 columns. The optical density would again be increased by light-scattering effects, and the actual amount of protein present would be negligible. Because of the interference of ampholine with the Lowry determination

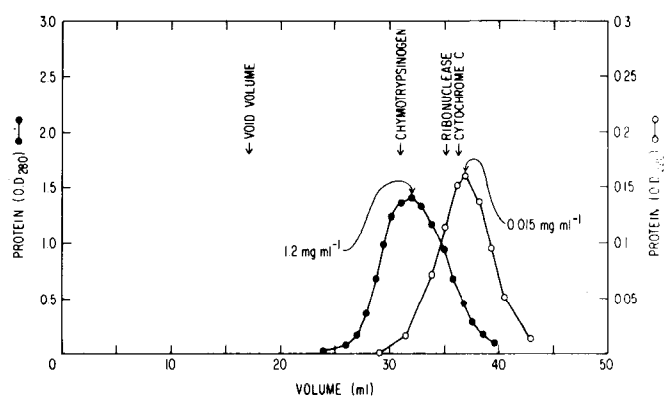


FIG. 3. Elution pattern of phospholipase A₂ at two concentrations on Sephadex G-100 column (1 × 50 cm). The sample volume was 1 ml in each case. The protein (Lowry method) concentration of the major fraction eluting for each sample is indicated. The void volume was determined with blue dextran 2000. Cytochrome c (11,700), ribonuclease (13,700), and chymotrypsinogen (25,700) were used to calibrate the column.

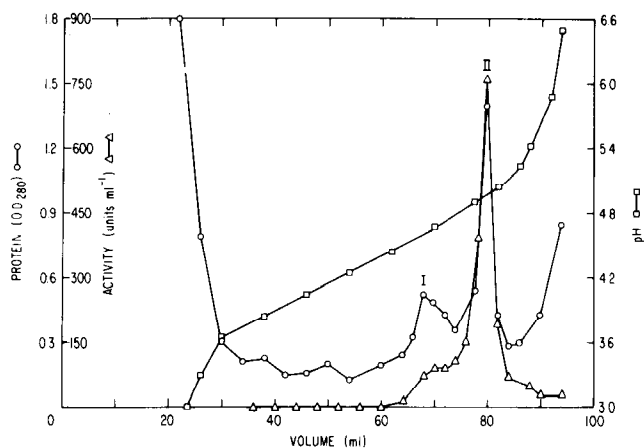


FIG. 4. Narrow range electrofocusing of 14 mg of phospholipase A₂ on a LKB 110-ml electrofocusing apparatus using LKB pH 4 to 6 ampholine. Experiments were conducted at 4° for 48 hours after equilibrium was reached and maintained at 1,000 volts and under 3 watts of power.

and the difficulty of completely removing it from the protein, it was not possible to determine accurately the protein concentration of the small peak so that this possibility could not be directly demonstrated.

It is also possible that the small peak observed on the isoelectric focusing represents protein in which the amide/acid ratios of the glutamate and aspartate residues differ slightly. Robinson *et al.* (19) have shown that some glutamine and asparagine residues may be particularly labile depending on the sequence and conformation of a protein. Currie *et al.* (20) have found that *Naja naja* venom gives two phospholipase A₂ peaks on DEAE-cellulose chromatography. They reported that the amino acid composition of the two peaks are identical, with the possible exception of the degree of amidation. This phenomenon together with the concentration-dependent aggregation discussed above and the problems encountered when attempting to reduce the enzyme's disulfide bonds, which are discussed below, may account for most, if not all, of the multiple forms of phospholipase A₂ reported to occur within a single venom sample (5, 10, 11).

It is interesting to note that Salach *et al.* (5) found, on isoelectric focusing of cobra venom, that the major phospholipase peak (which they designated Peak IIA) had an isoelectric point of 4.95 and was associated with a smaller peak of isoelectric point 4.75. This major peak had a molecular weight of 20,000, but was measured at protein concentrations greater than 0.2 mg ml⁻¹. Presumably, Salach *et al.* (5) separated the same form of the enzyme as reported here, but determined the molecular weight at a concentration in which the protein was aggregated.

Phospholipase A₂ Specificity—1-Palmitoyl,2-palmitoyl [9,10-³H]phosphatidylcholine was used as substrate in the normal assay system, but the reaction was allowed to proceed to completion. The products were chromatographed on thin layer chromatography plates with standards (8), and the pertinent spots were removed and counted in 2:1 Patterson-Greene counting solution. Ninety-seven per cent of the radioactivity chromatographed with the fatty acid peak, while the remaining 3% chromatographed with the lyso-phosphatidylcholine, proving that the enzyme is specific for the fatty acid at the C-2 position of the glycerol.

Amino Acid Composition—The results of the amino acid analysis are given in Table II. No free sulfhydryl groups could be detected by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (23), even in the presence of 6 M guanidine-HCl. Therefore, all of the ½ cystine residues are presumably present as five to six disulfide bonds. Of special interest is the finding of only 1 histidine and 1 tryptophan, because these residues are important in the study of the mechanism of action of the pancreatic enzyme (24) and preliminary experiments in our laboratory¹ have suggested their importance for the cobra enzyme as well.

Phospholipase A₂ Stability—The stability of phospholipase A₂, while beneficial when purifying and handling the enzyme, has also created some problems. The first was encountered when we attempted to run the enzyme on sodium dodecyl sulfate-polyacrylamide gels. If we used the standard Weber and Osborn technique (16), we not only found several bands but also found that the intensities of these bands were not always reproducible. Weber *et al.* (25) have discussed at length several factors that cause abnormal behavior on sodium

TABLE II
Amino acid composition of phospholipase A₂

Amino acid residue	Residues/molecule of enzyme ^a	Nearest whole no.
Asx	19.06	19
Threonine	4.32	4
Serine ^b	6.71	7
Glx	6.45	6
Proline	4.97	5
Glycine	9.15	9
Alanine	8.37	8
½ Cystine ^c	10.8	11
Valine	3.76	4
Methionine	0.70	1
Isoleucine	3.02	3
Leucine	4.73	5
Tyrosine	6.48	6
Phenylalanine	2.91	3
Histidine	0.93	1
Lysine	4.84	5
Arginine	3.80	4
Tryptophan ^d	1	1

^a Each figure represents an average of six determinations on a Beckman model 119 amino acid analyzer using the Beckman single column methodology. The results are based on a molecular weight of 11,000.

^b Obtained by extrapolation to zero time.

^c Determined on carboxymethylated enzyme (21).

^d Determined by *N*-bromosuccinimide procedure (22).

dodecyl sulfate gels. One of the most important is the incomplete reduction of the disulfide bonds and the concomitant decrease in the amount of sodium dodecyl sulfate bound to the protein. A second factor is that sodium dodecyl sulfate gel procedures become inherently more sensitive to various other perturbing factors when proteins of less than 15,000 g mol⁻¹ are run. The anomalous behavior found with this phospholipase A₂ is not surprising considering the existence of five to six disulfide bonds in a protein whose molecular weight is only 11,000. In both cases, the use of urea in the denaturing sodium dodecyl sulfate solution should minimize these problems. This was found to be true with this phospholipase A₂. With the use of 4 M urea and strong reducing conditions, reproducible gels were obtained which contained a single band. Thus, care must be taken before multiple forms of the enzyme can be inferred from the presence of more than one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

A second, related problem was encountered in measuring the ½ cystine content of the enzyme. We had first attempted this measurement by oxidation with performic acid, but found that one to two disulfide bonds were left intact unless unusually stringent conditions were employed. This procedure involved hydrolyzing the enzyme with 6 N HCl before treatment with performic acid. Unfortunately, quantitative yields were not obtained under the latter conditions. The reduction of the disulfide bonds with dithiothreitol in the presence of 6 M guanidine-HCl and their subsequent carboxymethylation did overcome this problem. Both the sodium dodecyl sulfate disc gel and the performic acid problems suggest that there is an area in the phospholipase, containing at least one disulfide bond, that is very resistant to unfolding. Since the enzymes from all sources studied thus far are relatively small and have a large number of disulfide bonds, it is important that attention be given to these problems when interpreting results from any

¹R. A. Deems and E. A. Dennis, unpublished experiments.

experiment that depends on the complete reduction of the disulfide bonds or denaturation of the enzyme.

REFERENCES

1. Pieterse, W. A., Vidal, J. C., Volwerk, J. J., and de Haas, G. H. (1974) *Biochemistry* **13**, 1455-1460
2. Wells, M. A. (1974) *Biochemistry* **13**, 2248-2257
3. Dennis, E. A. (1974) *J. Supramol. Struct.* **2**, 682-694
4. Cremona, T., and Kearney, E. B. (1964) *J. Biol. Chem.* **239**, 2328-2334
5. Salach, J. L., Turini, P., Seng, R., Hauber, J., and Singer, T. P. (1971) *J. Biol. Chem.* **246**, 331-339
6. Zwaal, R. F. A., Roelofsen, B., and Colley, C. M. (1973) *Biochim. Biophys. Acta* **300**, 159-182
7. Gatt, S., and Barenholz, Y. (1973) *Annu. Rev. Biochem.* **42**, 61-90
8. Dennis, E. A. (1973) *J. Lipid Res.* **14**, 152-159
9. Dennis, E. A. (1973) *Arch. Biochem. Biophys.* **158**, 485-493
10. Braganca, B. M., and Sambray, Y. M. (1967) *Nature* **216**, 1210-1211
11. Shiloah, J., Klibansky, C., and DeVries, A. (1973) *Toxicon* **11**, 481-490
12. Tsao, F. H. C., Cohen, H., Snyder, W. R., Kezdy, F. J., and Law, J. H. (1973) *J. Supramol. Struct.* **1**, 490-497
13. Braganca, B. M., Sambray, Y. M., and Ghadially, R. C. (1969) *Toxicon* **7**, 151-157
14. Deems, R. A., Eaton, B. R., and Dennis, E. A. (1975) *J. Biol. Chem.* **250**, 9013-9020
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
16. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412
17. Gilbert, L. M., and Gilbert, G. A. (1973) *Methods Enzymol.* **27**, 273-295
18. Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp. 356-357, John Wiley & Sons, New York
19. Robinson, A. B., Scotchler, J. W., and McKerrow, J. H. (1973) *J. Am. Chem. Soc.* **95**, 8156-8159
20. Currie, B. T., Oakley, D. E., and Broomfield, C. A. (1968) *Nature* **220**, 371
21. Blumenthal, K. M., and Heinrikson, R. L. (1972) *Biochim. Biophys. Acta* **278**, 530-545
22. Spande, T. F., and Witkop, B. (1967) *Methods Enzymol.* **11**, 498-505
23. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70-77
24. Volwerk, J. J., Pieterse, W. A., and de Haas, G. H. (1974) *Biochemistry* **13**, 1446-1454
25. Weber, K., Pringle, J. R., and Osborn, M. (1972) *Methods Enzymol.* **26**, 3-27

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