Purification and Properties of Sulfite Oxidase from Chicken Liver

PRESENCE OF MOLYBDENUM IN SULFITE OXIDASE FROM DIVERSE SOURCES*

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SUMMARY

Sulfite oxidase (EC 1.8.3.1) has been purified from chicken liver by modification of a procedure previously applied to bovine liver.

The enzyme appears homogeneous as judged by its behavior in sedimentation velocity experiments and polyacrylamide disc gel electrophoresis. A new activity stain for the enzyme on acrylamide gels is described.

Despite its apparent homogeneity, the enzyme consistently behaves in a heterogeneous manner in sedimentation equilibrium experiments, suggesting an associating system whose state of association depends on both enzyme concentration and anion content. The subunit molecular weight, determined by sodium dodecyl sulfate disc gel electrophoresis, is 55,000. The amino acid composition of the purified enzyme is reported.

The absorption spectrum of the enzyme reveals the presence of a $a_2$-like cytochrome, while chemical analysis and electron paramagnetic resonance (EPR) spectra indicate that molybdenum functions as a second prosthetic group.

The kinetics of the reaction of the enzyme with sulfite and cytochrome $c$ are consistent with a ping-pong mechanism.

The EPR signal from the molybdenum center of the enzyme displays a pH titration behavior similar to that of the bovine enzyme. In addition, the shape of the signal is extremely sensitive to the presence of anions. The heme EPR signal is very similar to that reported for cytochrome $b_5$.

The enzyme has also been partially purified from human liver, wheat germ, and Thiobacillus thioparus: each of these preparations displays a sulfite-dependent molybdenum EPR signal.

* This work was supported by Grant GM 00091 from the United States Public Health Service.
† Supported by Predoctoral Traineeship, Medical Scientist Training Program Grant GM 01678 from the National Institutes of Health.

MATERIALS AND METHODS

Frozen chicken livers were obtained from local supermarkets. Normal adult human liver was obtained at autopsy and was frozen at $-20^\circ$ until needed. Wheat germ was purchased from Pavo Co., Inc., Minneapolis. Thiobacillus thioparus (ATCC 8158) was grown on the medium of Baalsrud and Baalsrud (10) in 15-liter carboys at room temperature for 4 to 5 days with vigorous aeration through a sintered glass sparger. Under these conditions, it was found that satisfactory yield (1 to 2 g of cells per liter) could be obtained without readjusting the pH, and the indicator dyes were therefore omitted from the medium.

Sodium sulfite was purchased from Allied Chemical Co. Formaldehyde-bisulfite was prepared by Dr. H. J. Cohen of NIEHS, Research Triangle Park, N. C., as described previously (1). Potassium ferriyanaide was obtained from Mallinckrodt. Horse heart cytochrome $c$ (type III), human albumin, and ovalbumin were obtained from Sigma. Carbonic anhydrase from human erythrocytes was kindly provided by Dr. J. M. McCord of this department. Trypsin and catalase were purchased from...
Spectrometer, equipped with a 9.5 GHz microwave bridge as-
semble and operated at a modulation frequency of 100 kHz. A
Fabritek model 1072 instrument computer (Nicolet Instrument
in a Branson sonifier (model 517).

Md.), and destaining was conducted in the Quick Gel Destainer
of pH were made with a Radiometer type TTTl titrator, and a
for all routine centrifugations. Preparative ultracentrifugations
protein solutions were purchased from the Amicon Corp., Cam-
field sweep over the O-5000 gauss range.

A frequency counter and diphenyl picryl hydrazide were em-
Quartz tubes (120 mm x 3.2 mm, ID) were used for all samples.
noise reduction, base-line corrections, and integration of spectra.

0.01 mm ferricytochrome c, and 0.4 mM sodium sulfite in buffer
the total rate. Cytochrome c solutions were quantitated using
was followed in a Cary 14 recording spectrophotometer. A slow
was started by the addition of sulfite, and the initial change in
absorbance at 550 nm, versus a blank cuvette which lacked sulfite,
or in the presence of 1 mm to 10 mm sulfite. The solutions were anaerobic by the time the spectra were obtained.

Protein concentrations were determined either by their ab-
sorbance at 280 nm and 290 nm (14) or by the spectropho-
metric method of Murphy and Kies (15). Ammonium sulfate
ations were determined by the method of Green and Hughes

EDTA was included at a concentration of 0.1 mm in all buffer

Sulfite oxidase was assayed as described by Cohen and Frid-
ovich (1). The most commonly used assay involved the reduc-
tion of cytochrome c. Cuvettes contained 10 mm of enzyme, 0.01
0.4 mm sodium sulfite in buffer (Tris-Cl, 0.1 M, pH 8.5), a final volume of 2.5 ml. The reaction was started by the addition of sulfite, and the initial change in
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Acetone powder of chicken liver was prepared by the procedure
treatment of the avian enzyme, such as extraction of acetone powder, am-
monium sulfate precipitation, exposure to heat and fractionation
with acetone were essentially the same as those employed for the
purification of the beef liver enzyme (1). The chromatographic
techniques were, however, different, and are described below.

Ion Exchange Chromatography on DEAE-cellulose—The orange-
red solution was dialyzed against 0.005 M potassium phosphate,
buffer, pH 7.8, at 4° for 4 hours, with one buffer change after 2 hours.
The solution was then applied to a column (50 cm × 3.5 cm) of
DEAE-cellulose (Whatman DE-52) equilibrated at 4° with the
same buffer. The column was washed with this buffer until a
protein peak had been eluted. The enzyme, visible as a red band
at the top of the column, was then eluted with a linear gradient
of 0.005 M to 0.2 M potassium phosphate buffer, pH 7.8. A typi-
celution pattern from such a column is shown in Fig. 1. The
minor species of sulfite oxidase eluting with the major protein
peak, noted by Cohen and Fridovich (1), was only occasionally
seen in the chicken liver preparations.

Gel Exclusion Chromatography on Sephadex G-200—The peak
tions from the DEAE column were pooled and concentrated
to a final volume of less than 3 ml. This solution was then ap-
plicated to a column (90 cm × 4 cm) of Sephadex G-200, which had
been equilibrated at 4° with 0.1 M KC1 in 0.01 M potassium phos-
phate buffer, pH 7.8, and then chromatographed with the

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification factor</th>
</tr>
</thead>
</table>
| Potassium phospha-
tic extracts        | 4480   | 224,000       | 2,580,000   | 11.5             | 100   | 1.0               |
| Heating at 66°C   | 5130   | 185,700       | 2,470,000   | 12.6             | 96    | 1.1               |
| 50% ammonium sul-
flate precipitate   | 780    | 46,300        | 2,470,000   | 53.3             | 96    | 4.6               |
| 45 to 66% acetone precipitate | 500 | 6,000 | 1,050,000 | 180.0 | 42 | 15.6 |
| 50% ammonium sul-
flate precipitate   | 120    | 3,000         | 1,100,000   | 360.0            | 43    | 31.3              |
| Pooled fractions from DEAE-cellulose | 3 | 151.2 | 650,000 | 4190.0 | 24 | 362.0 |
| Gel fractionation on Sephadex G-200 | 2 | 41.0 | 250,000 | 6094.0 | 10 | 530.0 |

* Acetone powder, 510 g, as starting material.

* Includes fractions with A412/A590 of 0.60 to 0.78.
same buffer. Peak fractions from the effluent of this column were graded for purity by means of their absorbance at 413 nm and 280 nm. Fractions with an absorbance ratio ($A_{413}:A_{280}$) of 0.75 to 0.78 were pooled; this sample was used in the experiments which required the use of homogeneous enzyme. Fractions with a ratio of 0.60 to 0.75 were pooled and used in kinetic studies. Each pool was concentrated by ultrafiltration and frozen in 0.2 ml aliquots, which were individually thawed as needed.

**Purity of Chicken Liver Sulfite Oxidase**—Samples of enzyme obtained from the G-200 column were analyzed for purity by polyacrylamide disc gel electrophoresis, using the procedures of Jovin *et al.* (17), and of Orr *et al.* (18). The results are shown in Fig. 2. In 2A, enzyme of highest specific activity can be seen to appear homogeneous at the lower pH, but to contain a trace contaminant in the Tris-glycine gel system. Fig. 2B, in which a larger amount of enzyme was applied to each gel, shows a single trace contaminant in the purified preparation; increasing amounts of this trace band are also visible in the enzyme samples of lower specific activity. Other preparations of purified enzyme contained no detectable contaminant at this concentration. In 2C, the correspondence of the protein band with material staining positively for heme and also with sulfite-ferricyanide oxidoreductase activity is demonstrated.

The purity of the sulfite oxidase preparation was also confirmed by sedimentation velocity studies at 59,780 rpm. At a protein concentration of 6 mg per ml only a single, symmetrical peak was observed, with $s_{20,w}$ of 6.0. However, when the calculation of $s_{20,w}$ was attempted by performing the same experiment with lower concentrations of enzyme (1.8 to 4 mg per ml) the peak was no longer symmetrical, indicating that some dissociation of the enzyme into its subunits was probably occurring. While the reasons for this apparent dissociation have not yet been studied in detail, similar behavior was observed during sedimentation equilibrium experiments, as discussed below.

**Molecular Weight and Subunit Structure**—Sedimentation equilibrium ultracentrifugation runs were carried out in double sector cells, using either sapphire windows and interference optics or quartz windows with absorbance optics. Both the meniscus-depletion method of Yphantis (21) and the long column meniscus-depletion technique of Chervenka (22) were used. Experiments were performed in several different solvent systems, using 0.1 M NaCl, 0.05 M potassium phosphate, pH 7.8, or 0.1 M NaCl in 0.01 M potassium phosphate, pH 7.8. In all cases, the plotted data yielded an upward-curving line, indicative of size heterogeneity. Since electrophoretically homogeneous enzyme was used for the electron micrographic staining technique of Ogawa *et al.* (20).

On removal from the tube, the gel was immersed in a solution of buffer (0.01 M Tris-Cl, pH 8.5) containing formaldehyde-bisulfite (0.01 M), potassium ferricyanide (0.01 M), and copper sulfate (0.04 M) for 30 min at room temperature. It was then rinsed with water and placed in a solution of copper sulfate (0.01 M) in the same buffer for an additional 10 min, after which a gray-brown band of precipitated copper ferrocyanide was visible at the site of enzyme activity. Control experiments indicated that this reaction was dependent on the presence of sulfite, and that only a single band with the mobility of sulfite oxidase could be stained in an impure preparation containing at least 10 distinct protein bands.
FIG. 3. Sedimentation equilibrium ultracentrifugation of electrophoretically homogeneous sulfite oxidase in potassium phosphate, 0.05 M, pH 7.8, carried out according to Chervenka (22) at 20.8° and 14,290 rpm, using Rayleigh interference optics. An enzyme solution (50 μl) containing 0.7 mg of protein per ml was used. The bottom of the solution column was at r² = 50.95. Using the dashed line, a molecular weight of 108,000 was determined assuming a partial specific volume of 0.73, calculated from the amino acid composition (23).

these studies, the most likely explanation for this result is the establishment of an equilibrium between two enzymatic species, monomer and dimer, with the latter being the predominant species. Weight-average molecular weights at the bottom of the solution column calculated from these data ranged from 89,500 in 0.1 M NaCl to 108,000 in potassium phosphate buffer. The plotted data from the latter experiment are shown in Fig. 3. Preliminary data from experiments in this same solvent, but at initial protein concentrations approximately one order of magnitude lower (0.07 mg per ml), give a calculated weight-average molecular weight at the bottom of the solution column of approximately 87,000, indicating that both the concentration of the enzyme and the ionic composition of the buffer play a role in influencing the apparent monomer-dimer equilibrium.

Polyacrylamide disc gel electrophoresis of purified enzyme in the presence of sodium dodecyl sulfate was carried out by the method of Weber and Osborn (24), using transferrin (subunit molecular weight, 77,000), human serum albumin (67,500), catalase (60,000), ovalbumin (43,000), carbonic anhydrase (29,000), trypsin (23,000), and bovine superoxide dismutase (16,500) as standards. In the presence of mercaptoethanol, a single band was observed, which in Fig. 4, corresponded to a subunit molecular weight of 55,000. In a separate experiment, the molecular weight of the enzyme was shown to be identical in both the presence and absence of mercaptoethanol. Native sulfite oxidase therefore appears to be composed of two subunits, of equal size within limits of detection, which are capable of associating by noncovalent forces.

Amino Acid Analysis A solution containing 2 mg of purified sulfite oxidase was prepared for amino acid analysis by removal of the heme prosthetic group, using the acetone-HCl procedure applied previously to cytochrome b₅ by Oszols (25). Portions of the resulting apoenzyme were then hydrolyzed in vacuo with 6 N HCl, 0.1% phenol, for 24, 48, and 72 hours at 110°. Additional samples were treated with performic acid (26) prior to hydrolysis or were hydrolyzed in 6 N HCl with 4% mercaptoacetic acid (27). The results are presented in Table II. A remarkable feature is the unusually high content of proline (7.9%).

Prosthetic Groups—The absorption spectrum of purified chicken liver sulfite oxidase is shown in Fig. 5. As with the bovine enzyme, it closely resembles the spectrum of cytochrome b₅ (28) and shows no evidence of any prosthetic group other than the cytochrome. The heme of this preparation was shown to be identical to protoporphyrin IX by conversion to the pyridine hemochromogen according to the method of Falk (29). Quantitation of the heme content, on the basis of the molar extinction...
ferricyanide, or molecular oxygen as an electron acceptor. The reaction was also observed with the chicken liver enzyme. As with all previously reported preparations of sulfite oxidase (1, 13), the reaction of the chicken liver enzyme with cytochrome c was severely inhibited in the presence of anions.

The biphasic nature of the reaction with oxygen, noted earlier (1, 2), of sulfite oxidase was found to be capable of using cytochrome c, ferricyanide, or molecular oxygen as an electron acceptor. The initial rate of change in absorbance at 550 nm, versus a blank containing both substrates but without enzyme, was recorded. Dissolved oxygen did not interfere with these assays, since under these conditions osygen coefficients for the α-band of reduced minus oxidized pyridine hemochromogen, yielded a value of 1 mole of heme per 64,000 g of protein, or 0.8 mole of heme per subunit of molecular weight 56,000.

Molybdenum was assayed chemically by the method of Clark and Axel (30), and a value of 0.8 mole of molybdenum per subunit was obtained.

Catalytic Properties and Kinetic Parameters—Chicken liver sulfite oxidase was found to be capable of using cytochrome c, ferricyanide, or molecular oxygen as an electron acceptor. The biphasic nature of the reaction with oxygen, noted earlier (1, 2), was also observed with the chicken liver enzyme. As with all previously reported preparations of sulfite oxidase (1, 13), the reaction of the chicken liver enzyme with cytochrome c was severely inhibited in the presence of anions.

A two-substrate kinetic analysis was performed at 25° in 0.1 M Tris-Cl, pH 8.5, in a Cary 14 recording spectrophotometer using cuvettes of 10 cm light path. The initial rate of change in absorbance at 550 nm, versus a blank containing both substrates but without enzyme, was recorded. Dissolved oxygen did not interfere with these assays, since under these conditions oxygen is only 6% as effective an acceptor as cytochrome c. Furthermore, independent experiments using the oxygen uptake assay (1) established that the presence of cytochrome c prevents the reduction of oxygen by the enzyme. The kinetic results, shown in Fig. 6, are consistent with a ping-pong type of mechanism (31).

From these data, it was possible to calculate the true $K_m$ for sulfite $(2.4 \times 10^{-5} \text{M})$ and for cytochrome c $(2.2 \times 10^{-4} \text{M})$. These values may be compared with the values of $2.2 \times 10^{-5} \text{M}$ and $3.1 \times 10^{-7} \text{M}$, respectively, reported by Howell and Fridovich (13) for the bovine enzyme.

Characteristics of EPR Signals of Chicken Liver Sulfite Oxidase—Purified sulfite oxidase was studied with respect to its EPR properties. As in the case of the bovine enzyme (8), a signal was seen on reduction of the enzyme with sulfite. The appearance of this signal at $g = 1.97$, as well as its shape, identified it as the signal of Mo(V).1 No signal could be detected in the oxidized enzyme under the usual conditions of pH (7.8 to 8.5), ionic strength $(0.005 \text{M to } 0.05 \text{M Tris-Cl or potassium phosphate})$, and enzyme concentration (approximately $10^{-4} \text{M}$).

Cohen et al. (3) found that the molybdenum signal of bovine hepatic sulfite oxidase exhibited hyperfine splitting due to a dissociable proton. The influence of this proton on the signal could be eliminated either by titration with base or by equilibration with D2O. This was also the case with the chicken liver enzyme, as shown by the titration data of Fig. 7, and by experiments (not shown) in which equilibration with D2O eliminated the two major splittings in the spectrum at pH 7.0.

In addition to the disappearance of the hyperfine splitting due to the dissociable proton, the signals in Fig. 7 also show a general change in shape, indicating a change in the ligand field of the molybdenum from axial symmetry to lesser symmetry as the pH is raised. Since anions are known to exert considerable effects on the activity of the enzyme, the possibility that part of this change in signal shape might be due to the effects of buffer anions was investigated. Figs. 8 and 9 illustrate the results of some preliminary studies. The shape of the spectrum in the presence of chloride $(8 \text{A})$ is the same as that shown for pH 6.5 in Fig. 7, since nearly the same buffer was used. In Fig. 8, however, the conversion to a form resembling the pH 8.5 spectrum was accomplished not by changing the pH, but by the addition of phosphate. That the resulting signal is truly asymmetric was shown by the fact that equilibration with D2O failed to convert the high field doublet to a singlet. That the effect of phosphate and of pH are at least partially independent is shown by the fact that the central $g$ value, which shifts upfield when the pH is raised, remains unchanged after the addition of phosphate. The disappearance of the low field doublet, and its replacement by a slightly higher field singlet, however, occurs with both types of treatment.

Fig. 9 shows the results of the reciprocal experiment, in which a

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1 The abbreviation used is: Mo(V), pentavalent molybdenum.
FIG. 7. pH titration of the Mo(V) signal of chicken liver sulfite oxidase in the presence of sulfite (0.01 M) in 0.05 M Tris-Cl buffers of pH 6.5, 7.5, and 8.5. The arrows at the top of the figure indicate the direction of change in each peak as the titration progresses from pH 6.5 to pH 8.5. EPR conditions were as follows: microwave frequency, 9.109 GHz; microwave power, 5 milliwatts; modulation amplitude, 2 gauss; time constant, 1.0 s; gain, 4000; temperature, −100°C; and scan rate, 50 gauss per min.

The phosphate spectrum was converted into a typical Tris-Cl pH 7.0 spectrum by the addition of KCl. The possibility that the changes in signal shape shown in Figs. 8 and 9 are due to ionic strength, rather than specific anion effects is ruled out by the fact that spectra 8A (μ = 0.05) and 9C (μ = 0.37) are identical, as are 9A (μ = 0.11) and 8C (μ = 0.94). Furthermore, a spectrum identical to 8A is obtained in Tris-Cl buffer, pH 7.0, over the entire concentration range from 0.025 M (μ = 0.024) to 0.5 M (μ = 0.49); similarly, spectrum 9A does not change while the concentration of potassium phosphate, pH 7.0, is varied from 0.005 M (μ = 0.01) to 0.15 M (μ = 0.34). A more comprehensive study of the effects of anions on the Mo(V) EPR signal, the activity, and the physical and spectral properties of chicken liver sulfite oxidase is currently in progress.

Because the absorption spectrum of sulfite oxidase isolated from both chicken and bovine livers so closely resembles that of cytochrome b₅, an attempt was made to visualize the heme EPR signal of the chicken liver enzyme and to compare its properties to those reported for the heme signal of cytochrome b₅ by Bois-Poltoratsky and Ehrenberg (32). Fig. 10 shows the low spin heme signal obtained from the oxidized enzyme. While the general signal shape is very similar to that of the spectra reported for cytochrome b₅, only the middle g value shows a close correspondence with that of cytochrome b₅. The low and high field g values of this pH 8.5 spectrum, however, appear at positions intermediate between those reported for cytochrome b₅ at pH 7.0 and pH 11.54. In addition, the sulfite oxidase spectrum shows no trace of the high spin component reported for cytochrome b₅. The weak signal seen in sulfite oxidase at around g = 1.07 is caused by the molybdenum prosthetic group. That it is not a part of the heme signal was established by temperature and power saturation studies which showed that this signal was not diminished under conditions where the low spin heme signal was completely abolished. Both the cytochrome b₅ and sulfite oxidase spectra are markedly different from those reported for low spin hemins with sulfur ligands (33).

Detection by EPR of Molybdenum as a Component of Sulfite Oxidases from Other Species—Having established that both a...
The purification of sulfite oxidase from chicken liver has been achieved by a series of steps similar to those used for the bovine enzyme. In addition, the success with which this basic purification scheme has been applied to human liver by following the procedure for bovine liver (1) makes it likely that the enzyme can be purified from, at least, most mammalian sources by techniques identical in outline to those first used with bovine liver. Preliminary results indicate, however, that purification of the enzyme from plant and bacterial sources will have to follow a somewhat different procedure. The fact that a homogeneous protein can be isolated from chicken liver by this purification scheme, and that the enzyme so obtained is a molybdo-hemoprotein capable of reacting with molecular oxygen, corroborates the recent clarification of the properties of the bovine enzyme by Cohen et al. (13).

Nearly all the studies reported here have demonstrated a great degree of similarity between the properties of the bovine and chicken liver sulfite oxidases. Some interesting differences in behavior have been noted, however. While anions are inhibitory for the reaction of both enzymes with one-electron acceptors such as ferricyanide and cytochrome c, only in the avian enzyme was an apparent effect of anions on the sedimentation behavior of the enzyme noted. The relationship of this effect of anions on physical behavior to their inhibition of activity, as well as the significance of the anion effects on the EPR signal of molybdenum shown in Figs. 8 and 9, is currently under investigation. If it is true that dissociation of the enzyme into subunits is favored by low enzyme concentration, as well as by the presence of certain anions, as current data suggest, then it seems likely that the subunit itself is the active species, since the enzyme is routinely assayed at concentrations at least two orders of magnitude lower than the lowest concentration used in sedimentation equilibrium experiments reported here. It may be possible to verify the catalytic competence of the subunit by performing sedimentation velocity studies in the presence of substrates, using absorbance optics.

The similarities between sulfite oxidase and cytochrome $b_5$, first noted with the bovine enzyme, have been both confirmed and extended by the work reported here. While no conclusions concerning a possible intracellular relationship between these two proteins have so far been reached, recent studies localizing the site of sulfite oxidase activity to the intermembrane compartment of mitochondria (34-36) indicate that it is the mitochondrial, rather than the microsomal, cytochrome $b_5$ with which sulfite oxidase must be compared. One approach to the clarification of the relationship among sulfite oxidase and cytochromes $b_5$ from mitochondria and microsomes would be a comparison of their tryptic peptides, but this has not yet been attempted.

From an evolutionary standpoint, the most intriguing result reported here is the finding that the sulfite oxidases from such diverse species as man, cow, chicken, wheat, and $T$. thioparus all contain a functionally active molybdenum. With regard to human metabolic disease, this result permits several alternative explanations for the proposed defect in the one reported case of sulfite oxidase deficiency (37), since a deficiency of molybdenum, or of a hypothetical molybdenum-carrying protein, could have been distinguished from a defect in the enzyme itself merely on the basis of activity measurements.

Neither the wheat germ nor the bacterial enzyme has so far been isolated in a state of purity which would make it possible to detect a hemoprotein group. Lyric and Suzuki (7) did not specifically assay for molybdenum in their preparation of the $T$. thioparus enzyme, since at that time there was no reason to suspect the functional importance of molybdenum in sulfite oxidase activity. It is conceivable, however, that the purity of their preparation was overestimated and that a reported cytochrome contaminant was in reality the sulfite oxidase itself. If the $T$. thioparus and wheat germ sulfite oxidases contain molybdenum, it may be surmised that the critical catalytic function of the enzyme dicted that it remain remarkably constant in its properties throughout the stages of evolution. The implication of this result would be that the detoxification of sulfite is of more importance to an organism than has heretofore been suspected. A study of the comparative enzymology of sul-
fite oxidases from diverse sources should therefore prove profitable; our efforts are currently aimed in this direction.

Acknowledgments—The authors wish to thank Dr. James S. Huston for his advice and guidance in performing the analytical ultracentrifugation studies, and Dr. Howard Steinman for assistance in the amino acid analysis.

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