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Studies of the Hydroxylamine Metabolism of *Nitrosomonas europaea*. II. Molecular Properties of the Electron-Transport Particle, Hydroxylamine Oxidase*

Michael K. Rees

ABSTRACT: Hydroxylamine oxidase is the initial acceptor of electrons from hydroxylamine, one of the energy substrates for the chemoautotroph, *Nitrosomonas europaea*.

Hydroxylamine oxidase lacks terminal oxidase activity, and in order to obtain measureable turnover of the enzyme, the presence of an added terminal electron-acceptor compound is essential. In this paper, some of the physical, chemical, and enzymatic features of hydroxylamine oxidase have been studied. Sedimentation velocity experiments indicate that the mass of homogeneous hydroxylamine oxidase is close to 200,000 g/mole and that its sedimentation coefficient is 10 S. Difference spectroscopy studies performed at the temperature of liquid nitrogen demonstrate that the enzyme contains both *b*- and *c*-type cytochromes. Neither flavin nor carbon monoxide combining components are present.

In the preceding paper (Rees, 1968), a procedure was described for isolating highly purified solutions of the hydroxylamine-oxidizing enzyme of *Nitrosomonas europaea*, hydroxylamine oxidase. Information was presented which indicates that the terminal oxidase of the bacterium is not physically associated with hydroxylamine oxidase, and that, as a consequence, an added terminal electron acceptor is essential for turnover of the enzyme. Further, it was shown that the product of hydroxylamine oxidation varies with the electron-acceptor compound employed; for example, although nitrite is not formed in the presence of added mammalian cytochrome *c*, as much as 70% of the oxidized hydroxylamine is accounted for as the expected physiological product, nitrite, when phenazine methosulfate is used. In this paper, some of the physical and chemical characteristics of hydroxylamine oxidase have been studied.

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Experimental Procedure

Reagents. D_2O (at least 99.5%) was obtained from Matheson Coleman and Bell. All chemicals were reagent grade and were prepared as previously described (Rees, 1968). Hydroxylamine oxidase was isolated by the method outlined in Table V of the first paper of this series (Rees, 1968) and was routinely stored at 0°.

Sedimentation Analysis. The Spinco Model E analytical ultracentrifuge was used for sedimentation velocity and sedimentation equilibrium experiments. All studies were performed with the absorption optical system of the instrument together with a split-beam photoelectric scanner and monochromator (Beckman Instruments, Inc.). The optical system was aligned by the method of Schachman *et al.* and the camera lens was focused upon an air–liquid meniscus using off-axis illumination (Schachman *et al.*, 1962). Radially oriented masks (4 × 20 mm) were mounted upon both collimating and condensing lenses to minimize stray light arising from the various optical components. Monochromatic light of wavelength 410 mμ was selected so that very dilute solutions of hydroxylamine oxidase could be examined. The monochromator and photo-

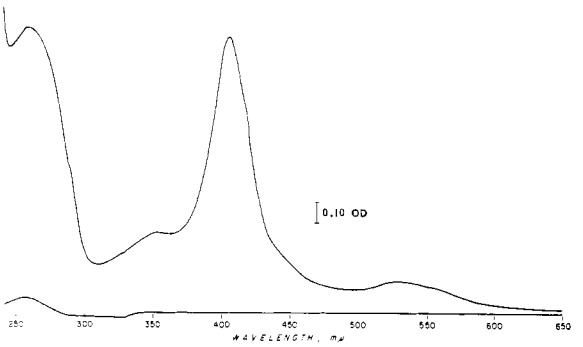

FIGURE 1: Absolute absorption spectrum of hydroxylamine oxidase, 0.8 mg of protein/ml. Solvent, 0.1 M phosphate buffer (pH 7.3); temperature, 25°.

multiplier slit widths were set at 2.0 and 0.1 mm, respectively.

Sedimentation equilibrium studies were performed by the high-speed method at 4° with a rotor velocity of 14,000 rpm. Liquid columns (3 mm) were employed and perfluorotributylamine (FC-43, Minnesota Mining and Manufacturing Co.) was added to the solution channel. The slow scan rate was used (6 min to traverse the image) and smooth traces were obtained by suitable adjustment of the electronic filter. To establish the time required for sedimentation equilibrium, traces taken at intervals were superimposed.

The value of the partial specific volume of hydroxylamine oxidase was determined by the sedimentation equilibrium method described by Edelstein and Schachman (1967). With this procedure, the parallel determination of the concentration distribution of a solute in solutions of H_2O and D_2O at sedimentation equilibrium permits the evaluation of both the partial specific volume and molecular weight. The partial specific volume is calculated from the equation

$$\bar{V} = \frac{k - [(\mathrm{d} \ln c/\mathrm{d}r^2)D_2O/(\mathrm{d} \ln c/\mathrm{d}r^2)H_2O]}{\rho D_2O - \rho H_2O[(\mathrm{d} \ln c/\mathrm{d}r^2)D_2O/(\mathrm{d} \ln c/\mathrm{d}r^2)H_2O]} \quad (1)$$

where k is the ratio of the molecular weight of the protein in the deuterated solvent to that in the nondeuterated solvent; thus, k serves to correct for the increase in molecular weight resulting from deuterium exchange.

To employ eq 1, it is necessary to perform the two sedimentation equilibrium experiments at the identical rotor speed and temperature, so that the solvent density is the only variable. In the present study, these conditions were fulfilled by diluting hydroxylamine oxidase (20 μg of protein/ml) into both H_2O and D_2O solutions (0.1 M Tris-HCl, pH 7.3 at 25°) and performing simultaneous high-speed sedimentation equilibrium experiments with two double-sector cells (aluminum-filled epon, 12-mm diameter, sapphire windows) and the multiplex operation of the scanner (Schachman and Edelstein, 1966). Solvent densities at 4° were determined pycnometrically. The concentration distribution at sedimentation equilibrium was determined by plotting the logarithm of the optical density (λ 410 mμ) as a function of the square of the distance from the center of rotation, taking points at 1-mm intervals (trace coordinate). Once the partial specific volume has been determined, the apparent weight-average molecular weight is calculated from

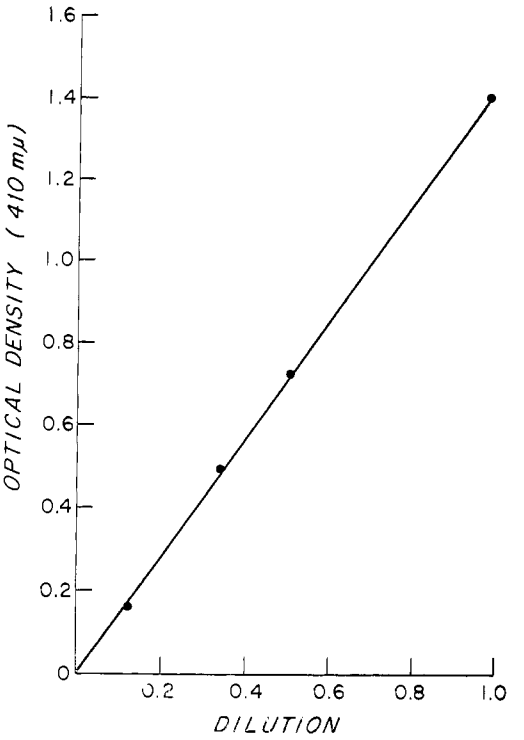

FIGURE 2: Linearity of the scanner recording system as a function of concentration of hydroxylamine oxidase. Solutions of hydroxylamine oxidase were examined at λ 410 mμ with a double-sector cell (12-mm path length) and with water as the reference liquid. All measurements were made at 14,000 rpm with the monochromator and photomultiplier slit widths set at 2.0 and 0.1 mm, respectively.

$$M_{w,app} = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}r^2} \quad (2)$$

The number-average and weight-average molecular weights of hydroxylamine oxidase were also determined. For these measurements, the enzyme (43 μg of protein/ml) was dissolved in 0.1 M Tris-HCl (pH 7.3) and examined by the high-speed equilibrium method outlined above. Point weight-average and point number-average molecular weights were evaluated from eq 3 and 4. Values of $M_{w(r)}$ were calculated from the slope of

$$M_{w(r)} = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}r^2} \quad (3)$$

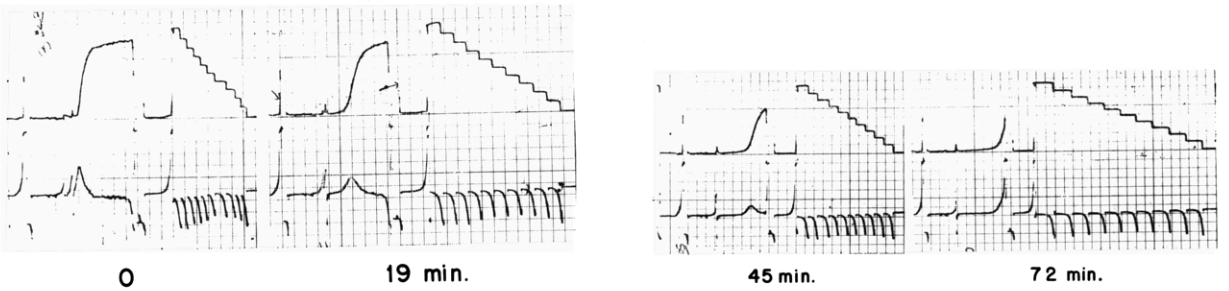

FIGURE 3: Sedimentation velocity patterns of a dilute solution of hydroxylamine oxidase (0.8 mg of protein/ml). The time after reaching 60,000 rpm is shown below each pattern. Sedimentation is to the right. The upper trace is the function curve, and the lower trace is the derivative curve. Each stairstep at the right of the function trace is equivalent to 0.2 OD unit. Monochromatic light of wavelength 410 mμ was used.

$$M_{n(r)} = \frac{c_r}{\frac{(1 - \bar{V}\rho)\omega^2}{2RT}\int_{r_m}^{r} c_r dr^2} \qquad (4)$$

the least-squares line fitted to each set of five adjacent points (Yphantis, 1964). The integral in eq 4 was estimated by trapezoidal summation. A PDP-1 digital computer was used for data reduction and M_n and M_w at infinite dilution of the enzyme were obtained from plots of the reciprocal of the apparent molecular weight as a function of c_r. Only values of c_r greater than 0.06 OD unit (λ 410 mμ) were used in this extrapolation.

Sedimentation velocity studies were performed at 4° with a rotor velocity of 60,000 rpm using both the integral and derivative circuits of the scanner. To obtain sharp derivative curves, a 6-sec scanning period was employed.

Electron Microscopy. Electron microscopy was performed with an RCA EMU-3 electron microscope at a magnification of 46,000×. Samples were dialyzed against 0.1 M NH_4OAc in triple-distilled H_2O, sprayed onto 200-mesh carbon-covered grids, dried, and shadowed with platinum at an angle of 1:6.

Protein Concentration. The concentration of protein was determined by the Lowry method with a bovine serum albumin standard (Lowry *et al.*, 1951).

Assay of Electron-Transfer Carriers. Absorption spectroscopy at room temperature was performed with a Cary 15 recording spectrophotometer and 1-cm path length quartz cuvets. At the temperature of liquid nitrogen, absorption spectroscopy was performed with a split-beam Johnson Research Foundation spectrophotometer (Wilson, 1967). For these studies, cuvets (diameter = 2 mm) similar to those described by Bonner (1961) were used. The cuvets were first cooled to the temperature of liquid nitrogen, and the sample was then quickly injected *via* a syringe. All samples were scanned at least three times to ensure the reproducibility of the measurements.[1] Fluorescence measurements were performed at room temperature with an Aminco-Bowman fluorometer.

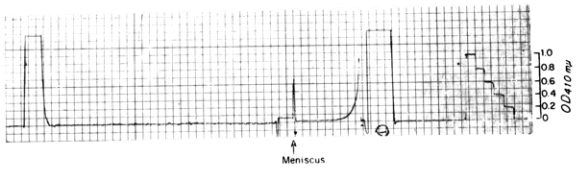

FIGURE 4: High-speed sedimentation equilibrium patterns of hydroxylamine oxidase. The trace was recorded with light of wavelength 410 mμ after 15 hr of centrifugation at 14,000 rpm. Solvent, 0.1 M Tris-HCl, pH 7.3; protein concentration, 20 μg/ml. For illustration purposes, a medium scan speed was used.

Results

All sedimentation analyses of hydroxylamine oxidase were performed with monochromatic light of wavelength 410 mμ. This represents the wavelength of maximum absorption of the oxidized enzyme in the Soret region of the spectrum (see Figure 1). To establish the linearity of the recording system of the scanner as a function of enzyme concentration, solutions of hydroxylamine oxidase of known dilution and optical density were examined in a double-sector cell with distilled H_2O as the reference liquid. Under the conditions of this study, Beer's law is followed to at least 1.2 OD units as Figure 2 demonstrates.

Figure 3 shows a series of patterns from a sedimentation velocity experiment with a dilute solution of hydroxylamine oxidase (0.8 mg of protein/ml). The calculated sedimentation coefficient (corrected to the viscosity of H_2O at 20°) is 10.2 S. The sedimentation coefficient was also determined after concentrating the enzyme by ultrafiltration to 5 mg of protein/ml. With the schlieren optical system, $s_{20,w}$ = 10 S was obtained. This information indicates that the infinite dilution value of the sedimentation coefficient of hydroxylamine oxidase is close to 10 S.

[1] I wish to express my gratitude to Dr. David Wilson of the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa., for his kind assistance in obtaining these low-temperature spectra.

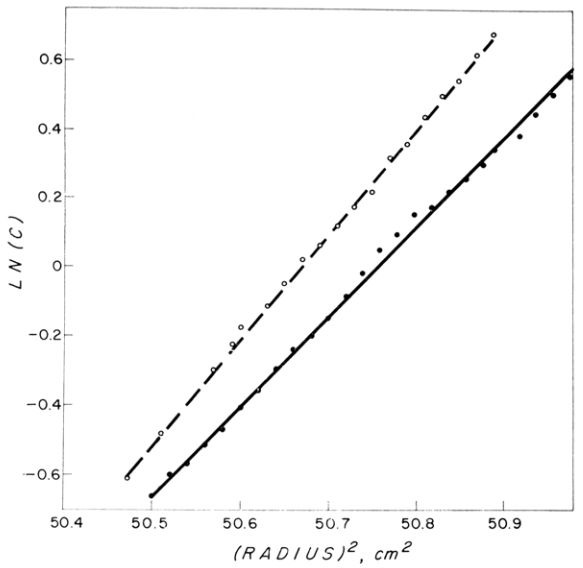

FIGURE 5: Plots of the logarithm of the concentration as a function of the square of the distance from the axis of rotation for hydroxylamine oxidase (20 μg of protein/ml) dissolved in both H_2O (○—○) and 95% D_2O (●—●). All solutions contained 0.1 M Tris-HCl (pH 7.3). Rotor speed, 14,000 rpm; rotor temperature, 4°.

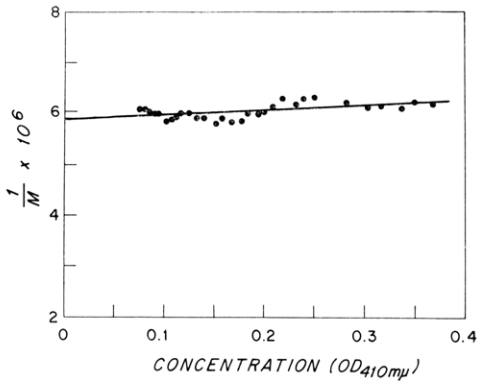

FIGURE 6: High-speed sedimentation equilibrium study of hydroxylamine oxidase as a function of concentration. Initial protein concentration, 43 μg/ml; solvent, 0.1 M Tris-HCl, pH 7.3; rotor temperature and velocity, 4° and 14,000 rpm, respectively (see Experimental Procedure for details).

A typical scanner trace at sedimentation equilibrium is shown in Figure 4, and Table I summarizes the measurements of the apparent partial specific volume and molecular weight of hydroxylamine oxidase determined by parallel sedimentation equilibrium measurements. The plots of the logarithm of the concentration as a function of the square of the distance from the axis of rotation for the enzyme dissolved in H_2O and in 95% D_2O are presented in Figure 5. To calculate the $\bar{V}$ of hydroxylamine oxidase from the slopes of these lines it is necessary to know the value of k in eq 1, since this term corrects for the increase in molecular weight due to deuterium exchange. Since the number of exchangeable hydrogens in hydroxylamine oxidase is not known, it has been assumed that this enzyme contains about the same number as has been experimentally determined for a variety of proteins. Thus, $k = 1.008$ was used in expt 1 (47.5% D_2O–H_2O pair) and $k = 1.0155$ in expt 2 (95% D_2O–H_2O pair) (Edelstein and Schachman, 1967). With these values of k, $\bar{V} = 0.653$ ml/g is calculated. Although this number may require revision in the future because of the uncertainty with regard to exchangeable hydrogens, this represents a relatively small correction, and it is doubtful that the

TABLE I: Simultaneous Determination of the Partial Specific Volume and Molecular Weight of Hydroxylamine Oxidase.[a]

Solvent Pair (%)	(d ln c/dr^2)D_2O/(d ln c/dr^2)H_2O	$\bar{V}$ (ml/g)	$M_{w,app}$
D_2O–H_2O (47.5)	0.928	0.665	202,000
D_2O–H_2O (95)	0.858	0.652	191,000
	Mean	0.65_3	196,000

[a] Solvent: 0.1 M Tris-HCl (pH 7.3) with the concentration of D_2O noted. Rotor temperature and velocity: 4° and 14,000 rpm, respectively. All solutions contained 20 μg of protein/ml.

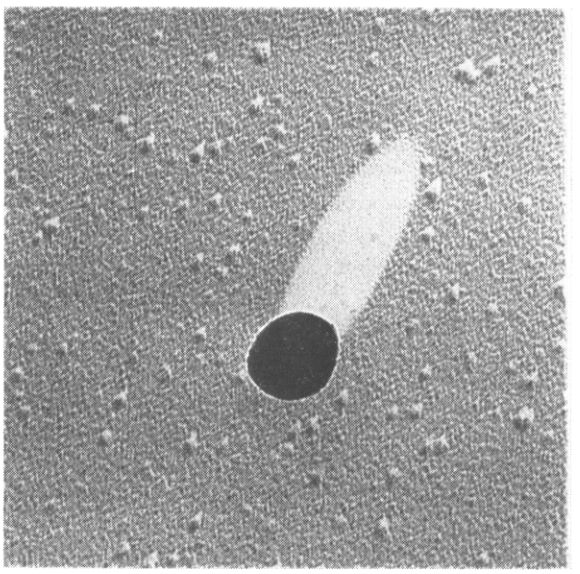

FIGURE 7: Electron microscopic appearance of platinum-shadowed (1:6) hydroxylamine oxidase. Magnification, 100,400×.

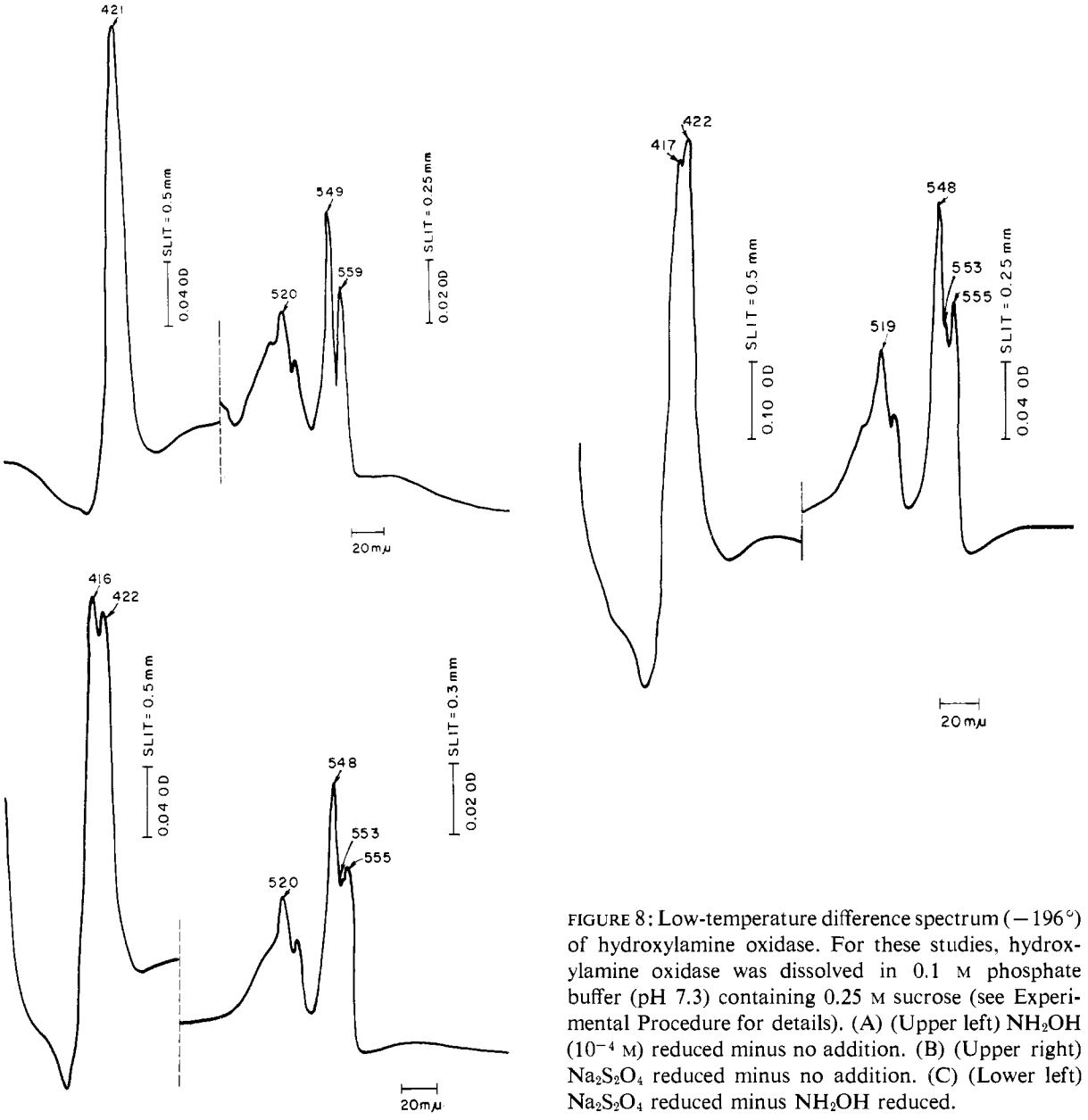

FIGURE 8: Low-temperature difference spectrum (−196°) of hydroxylamine oxidase. For these studies, hydroxylamine oxidase was dissolved in 0.1 M phosphate buffer (pH 7.3) containing 0.25 M sucrose (see Experimental Procedure for details). (A) (Upper left) NH_2OH (10^{-4} M) reduced minus no addition. (B) (Upper right) $Na_2S_2O_4$ reduced minus no addition. (C) (Lower left) $Na_2S_2O_4$ reduced minus NH_2OH reduced.

value will be altered significantly when k is known more precisely. With $\bar{V}$ = 0.653 ml/g, the apparent weight-average molecular weight of hydroxylamine oxidase is close to 196,000 g/mole (last column of Table I).

The above particle weight of hydroxylamine oxidase was verified from a determination of the number-average and weight-average molecular weights of a second preparation of the enzyme (43 μg of protein/ml, 0.1 M Tris-HCl, pH 7.3; see Experimental Procedure for details of these calculations). The plots of the reciprocal of the point average molecular weights as a function of concentration were essentially identical, and the data of the number-average molecular weight determination are summarized in Figure 6. The close agreement between the number-average and weight-average molecular weights (M_n = 204,000 and M_w = 216,000, respectively) when taken together with the linearity of the ln c *vs.* r^2 plots (Figure 5) and the symmetry of the sedimentation velocity curves (Figure 3) indicates that hydroxylamine oxidase possesses a high degree of particle size homogeneity and that its mass is about 200,000 g/mole.

Electron Microscopy. Although electron microscopic examination of the platinum-shadowed particle does not reveal significant detail, Figure 7 does indicate that hydroxylamine oxidase is essentially a spherical particle with a diameter of about 160 Å. With the knowledge that hydroxylamine oxidase is

a sphere, it is also possible to calculate the diameter from a knowledge of its sedimentation coefficient and molecular weight. For this calculation, the Svedberg equation and the equation relating the diffusion and frictional coefficient are used ($M = RT/D(1 - \bar{V}\rho)$ and $D = RT/Nf$, respectively) together with the equation for the frictional coefficient of a sphere ($f = 6\pi\zeta r$). Since for hydroxylamine oxidase, $s_{20,w}$ = 10 S and molecular weight = 200,000, the hydrodynamic diameter of the enzyme is calculated to be 122 Å. This number probably more closely reflects the true diameter of the particle than the value approximated from electron microscopic examination of the platinum-shadowed preparation.

Analysis of Electron-Transfer Carriers. To characterize the electron-transfer carriers of hydroxylamine oxidase, difference spectroscopy was performed at the temperature of liquid nitrogen. For these experiments, 0.25 M sucrose was added to the buffer, since this increases the sensitivity of the measurement (Wilson, 1967). Figure 8A presents the appearance of enzyme after steady-state reduction with substrate (1×10^{-4} M NH_2OH). A single peak is present in the Soret region at about 421 mμ, the major β peak is at 520 mμ, and two well-separated peaks at 549 and 557 mμ appear in the α region. Only about 50% of the total heme pigment present in hydroxylamine oxidase is reduced by substrate, since upon addition of solid sodium dithionite to a solution of enzyme previously reduced by substrate, all peaks are approximately doubled. This is demonstrated in Figure 8B, which also reveals that dithionite addition causes the appearance of additional reduced peaks at 417 and 553 mμ. To illustrate clearly the further reduction of cytochrome which follows dithionite treatment, the difference spectrum of dithionite-reduced minus hydroxylamine-reduced enzyme is presented in Figure 8C. None of these reduced pigments combines with carbon monoxide.

These spectra indicate that hydroxylamine oxidase contains at least one *b*- and one *c*-type cytochrome. The maximum extent of reduction of any cytochrome in the presence of hydroxylamine is about 50% of that which results upon reduction of the enzyme with dithionite. The appearance of peaks at 553 and 417 mμ (which are assigned to cytochrome *c*) only after treatment with dithionite may indicate that hydroxylamine reduces the cytochrome *c* of the particle to a lesser extent than it does cytochrome *b*.

Examination of hydroxylamine oxidase by absorption spectroscopy and by fluorimetry at room temperature failed to provide evidence for the presence of a flavin component, nor were flavins noted in acid extracts of the particle.

Discussion

In the first paper of this series (Rees, 1968) it was shown that highly purified solutions of hydroxylamine oxidase could be prepared which retain at least 40% of the total enzymatic activity present in the crude extract. The findings of the present study indicate that hydroxyl-

TABLE II: Composition of the Electron-Transport System of *N. europaea*.

Component	Temp (°C)[f]	Cytochrome	Absorption Max in Reduced Spectrum (mμ) γ	β	α	Absorption Max in CO-Combining Spectrum (mμ) γ	β	α	Mol Wt (g/mole)	$s_{20,w}$ (S)
Hydroxylamine oxidase	−196	*b*	422	?	555 + 557[a]		None		200,000	10
		c	417	520	549 + 553[a]					
Terminal oxidase	−196	*a*	430		595	446[b]		599[b]	128,000[d]	8[d]
	25	*o*		?		418[c]	540[c]	565[c]		
Fraction F_2[e]	25	c_{552}	420	523	552		None		20,000	

[a] The assignment of the fine structure in the α region is arbitrary. [b] The CO-combining complex of cytochrome *a* is dissociated by light at −196° (M. K. Rees, unpublished data). [c] Rees and Nason (1965). The CO complex of cytochrome *o* is not dissociated by light (M. K. Rees, unpublished data). [d] It is not known whether this is a property of cytochrome *a* or cytochrome *o* or a complex of the two cytochromes. [e] Rees (1968). [f] The temperature refers to the temperature of spectroscopic analysis.

amine oxidase exhibits a high degree of physical as well as chemical homogeneity and that it contains at least one *b*- and one *c*-type cytochrome. Electron microscopic examination reveals the enzyme to be a spherical particle, and a diameter of 122 Å is calculated from the experimentally determined values of its molecular weight (200,000 g/mole) and sedimentation coefficient ($s_{20,w}$ = 10 S).

The molecular properties of hydroxylamine oxidase which have been elucidated in this paper are summarized in Table II. Since considerable information is now available with regard to other components of the *Nitrosomonas* electron-transport system, these data have also been included in the table. To the author's knowledge, this is the first report of the existence of an electron-transport system which is entirely soluble in the absence of prior treatment with detergents or physical fragmentation procedures such as sonic oscillation.

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Glutamate Biosynthesis in Anaerobic Bacteria. III. The Origin of the Carboxyl Groups of Glutamic and Aspartic Acids Isolated from *Clostridium kluyveri* Grown on [1-^{14}C]Acetate*

Joseph R. Stern and R. W. O'Brien

ABSTRACT: Radioactive glutamic and aspartic acids were isolated by acid hydrolysis of the protein from whole cells of *Clostridium kluyveri* (Worthington strain) grown on [1-^{14}C]acetate and the distribution of ^{14}C was determined in each compound. With glutamic acid, 25.5% of the ^{14}C was in C-1, 36.7% in C-2 to -4, and 38.8% in C-5. With aspartic acid, 23.9% was in C-1, 49.1% in C-2 and C-3, and 27.0% in C-4. The glutamate results were consistent with those previously obtained with cell-free extracts of the same strain (Stern, J. R., Bambers, G., and Hegre, C. S. (1966), *Biochemistry 5*, 1119) and demonstrated that the stereospecificity of the citrate synthetase enzyme of the glutamate biosynthetic pathway was essentially the same in the intact cell as in extracts of this strain. It was shown that some of the [1-^{14}C]acetate was converted to $^{14}CO_2$, about 4% of the acetate radioactivity being recovered as $^{14}CO_2$ in the spent medium. This conversion accounted for the finding that both carboxyls of aspartate were approximately equally labeled. The radioactivity found in C-1 of glutamate was shown to have arisen mostly from $^{14}CO_2$ *via* [4-^{14}C]oxalacetate (aspartate) and the remainder (16%) by carbon translocation involving unknown reactions. The nature of the reactions leading from [1-^{14}C]acetate to $^{14}CO_2$ was not identified but may involve formation and decarboxylation of acetoacetate.

The pioneering work of Tomlinson (1954a) on the distribution of ^{14}C in the glutamic acid isolated from cells of *Clostridium kluyveri* grown in a synthetic medium containing $^{14}CO_2$ or [1-^{14}C]acetate demonstrated that the label was found mostly but not exclusively in the opposite carboxyl of glutamate to that predicted by the operation of the Krebs citric acid cycle. It has now been clearly established that glutamic acid biosynthesis in this microorganism does occur mostly, if not entirely, by a pathway involving the enzymes of the upper half of the citric acid cycle (Stern and Bambers, 1966; Gottschalk and Barker, 1966; Ilse and O'Brien, 1967). Gottschalk and Barker, using cell-free extracts, found a labeling pattern of glutamic acid

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