and a conservative cut of luciferase activity at the DEAE-Sephadex column step gives material having about half the specific activity of affinity purified enzyme (cut conservatively) with only a 17.5% yield from the initial lysate (Hastings et al., 1978). The affinity method was therefore judged to be of greater utility for small- or large-scale luciferase purification than the standard batch elution and ion-exchange methods.

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Raman Spectroscopy of Avidin: Secondary Structure, Disulfide Conformation, and the Environment of Tyrosine†

Richard B. Honzatko* and Robert W. Williams

ABSTRACT: An analysis of the amide I region of Raman spectra indicates that avidin has 10 ± 5% and 55 ± 4% of its residues in helical and β-strand conformations, respectively. Predictions of secondary structure on the basis of the sequence of avidin are consistent with the high percentage of residues in the β conformation. We observe no differences between the spectra of avidin in solution and in crystals nor is there a significant difference between the secondary structures of avidin and the complex of avidin with biotin. In addition, the ratio of the intensities of the tyrosine doublet at 826 and 855 cm^{-1} indicates the lone tyrosine side chain of an avidin subunit is in a strong hydrogen bond as a proton acceptor. The Raman data also indicate the single disulfide of an avidin subunit has dihedral angles of 0–50° for each of its two C_β–S bonds and a dihedral angle of 85 ± 20° for its disulfide bond. We discuss the significance of these results in relation to findings of earlier work on avidin.

Avidin from the whites of hen eggs is a tetramer composed of monomers of M_r 15700. Each subunit of avidin binds one molecule of D-biotin with an affinity (pK_d = 15) among the highest of all associations between naturally occurring ligands and biological macromolecules (Green, 1963). The biological role of avidin in the egg is not clear. Green (1975), however, has suggested an antibacterial function for avidin; by sequestering D-biotin, avidin inhibits bacterial growth in the egg. On the basis of binding studies of biotin analogues, the principal interaction of the biotin molecule with avidin involves only the ureido portion of the ligand. In addition, the property of D-biotin to protect tryptophan residues from oxidizing agents suggests the proximity of indole side chains to the binding

† From the Laboratory for the Structure of Matter (R.B.H.) and the Bio/Molecular Optics Section, Optical Probes Branch (R.W.W.), Naval Research Laboratory, Washington, DC 20375. *Received July 12, 1982.* R.B.H. and R.W.W. are National Research Council postdoctoral fellows.

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pocket for biotin. A thorough review of the physical and chemical properties of avidin is in the literature (Green, 1975).

In recent years, work on avidin has centered on exploiting the interaction of biotin and avidin as a multipurpose tool for the biochemist (Bayer & Wilchek, 1980). Avidin coupled to an appropriate support medium can be used in the isolation of proteins containing biocytin or derivatives of biotin (Berger & Wood, 1975). In addition, conditions exist for labeling proteins with biotin so as to leave unimpared the protein–receptor interactions at the cell membrane, while simultaneously allowing an avidin–ferritin couple to bind to the biotin-labeled protein. The ferritin molecule, because of its high opacity to electrons, marks the location of the membrane receptor in electron micrographs.

A better understanding of the structural properties of avidin may be of benefit in extending the interaction of biotin and avidin as a biochemical tool. Although much is known about avidin, definite information concerning the secondary and tertiary structure of the protein is absent. For instance, reliable quantitative estimates of secondary structure on the basis of the circular dichroism spectra of avidin were not possible in an early study (Green & Melamed, 1966). The early work predates the introduction of methods for the estimation of secondary structure from circular dichroism spectra. In addition, difficulties in reproducing avidin crystals of diffraction quality frustrated early crystallography efforts (Green & Joynson, 1970). Problems in crystal growth have been overcome only recently (Pinn et al., 1982).

In the present study we investigate the structural properties of avidin and its complex with biotin by Raman spectroscopy. Estimates of secondary structure on the basis of the amide I region of Raman spectra are possible with high certainty. In addition to the analysis of the amide I region, Raman spectroscopy provides information as to the local environment of the single tyrosine of the avidin subunit and the conformation about its single disulfide. The results of the Raman investigation are consistent with predictions of secondary structure on the basis of the sequence of avidin, as well as with the results of chemical modification studies of avidin in solution.

Materials and Methods

Avidin (binding capacity of 13.4 μg of biotin/mg of avidin), obtained from Sigma, was crystallized as rectangular plates (Green & Joynson, 1970) by dialyzing a protein solution (concentration of 15 mg/mL) against 70% saturating ammonium sulfate, pH 5.0. In order to avoid orientation effects of large single crystals in the Raman experiments, we chose batches of crystals that ranged in size from 0.02 to 0.04 mm. The crystals were well formed and optically clear to visible light. All other chemicals used in the experimental work were reagent grade aside from the ammonium sulfate, which was ultrapure grade from Schwarz/Mann. Our source of D-biotin was Sigma.

Crystals of avidin were washed in 70% saturating ammonium sulfate, pH 5.0, and then pelleted in a melting-point capillary. Solution spectra were taken on 3-μL volumes, which contained avidin dissolved in distilled water at a concentration of 10%. We determined the molar concentration of avidin by ultraviolet absorption (Green & Toms, 1970), taking the molecular weight of a monomer subunit as 15700. A 1.0 M solution of D-biotin prepared gravimetrically was added to a 5% excess over the number of binding sites.

The excitation source was a Spectra Physics Model 170 argon ion laser, consisting of 75 mm focal length f1 collection optics, a Spex 1401 double monochromater with gratings of 1800 grooves/mm, and an RCA 31034 photomultiplier tube cooled to −30 °C. The spectral bandwidth of the instrument for these experiments was 6.5 cm^{-1} at 17775 cm^{-1}. We operated the laser at 514.5 nm and 500 mW, focusing the beam to an approximately 0.9-mm diameter at the sample. All spectra were collected on samples cooled to 10 °C.

Repetitive scanning was under the control of a National Semiconductor RMC microcomputer coupled to a Chromemco microcomputer. The scanning rate was 0.5 cm^{-1}/s over a range of 1500–1800 cm^{-1} with data taken at every 1 cm^{-1}. We accumulated 40 scans for each spectrum, storing each scan separately on a magnetic disc. Spectra of a given sample were averaged, and the resulting spectrum was five-point smoothed by the algorithm of Savitsky & Golay (1967). Smoothing at an interval of five data points (5 cm^{-1}) does not eliminate information, because the band-pass of the instrument is slightly higher than 5 cm^{-1}.

We used the procedure of Williams & Dunker (1981) and R. W. Williams (unpublished results) to remove the contribution of solvent/buffer and the fluorescence background from the Raman spectra in the amide I region. Weighted buffer spectra were subtracted from Raman spectra to remove buffer peaks. In order to remove entirely the intense peaks due to ammonium sulfate in the sample of avidin crystals, we applied a slightly higher weight to the buffer spectrum than indicated by the criterion of linear extrapolation of Williams & Dunker (1981). A linear correction brought the base line of the Raman spectra (with buffer contributions removed) to approximately zero intensity. An additional nonlinear correction removed the remaining curvature of the base line due to fluorescence. On the basis of difference spectra of samples having high and low fluorescence, the best approximation to this curvature was a Gaussian curve centered at 1616 cm^{-1} with a standard deviation of 59.5 cm^{-1}. We fit Lorentz–Gaussian product functions to the remaining peaks of the Raman spectra outside the amide I region. These functions were subtracted from the spectra, leaving only the amide I band. Finally, the Raman spectra, adjusted as described above, were normalized according to the procedure of Williams & Dunker (1981). Noise levels in the spectra were on the order of 1–2% of the amide I maximum.

A full description of the procedure for estimating the percentage of secondary structure on the basis of the amide I band will appear elsewhere (R. W. Williams, unpublished results). Briefly, the analysis centers on minimizing the residual between a linear combination of the amide I spectra of a set of proteins whose secondary structures are known and the spectrum of the specimen protein. In other words, given a p by n matrix A, where each of n columns has p data points of an amide I spectrum of a protein of known secondary stucture and given a vector $\mathbf{b}$ of p data points of the specimen protein, then an n vector $\mathbf{x}$ must be found such that

$$A\mathbf{x} \simeq \mathbf{b} \tag{1}$$

Once the best $\mathbf{x}$ is found by a least-squares procedure, estimates of secondary structure are derived from

$$F\mathbf{x} = f \tag{2}$$

where F is an m by n matrix representing the frequency of m classes of secondary structure among the n proteins used in the solution of $\mathbf{x}$ and where f is the fraction of each of m classes of secondary structure in the specimen protein. In generating the elements of matrix F, we used the criteria of Levitt & Greer (1977) to analyze the secondary structure of proteins solved by X-ray diffraction techniques. For the work, here, spectra of 14 proteins used in the work of R. W. Williams (unpublished results) contributed to matrix A.

Table I: Secondary-Structure Content (%) of Avidin by Two Least-Squares Methods

avidin state	method[a]	structure type[b]						totals	
		H_o	H_d	S_a	S_p	T	U	H	B
crystals	R1	0	10	46	7	22	14	11	53
	R2	5	6	57	0	22	11	10	57
solution	R1	−1	11	48	5	22	14	10	53
	R2	4	5	58	0	22	10	9	58
biotin	R1	−3	8	52	3	23	16	4	55
	R2	6	7	56	0	21	11	11	56
standard deviation[c]	R2	5	4	5	3	3	3	6	4
	R2	5	4	5	4	3	4	5	3

[a] (R1) Equation 1 was solved with subprogram SVA (Lawson & Hanson, 1974), which allows negative solutions; (R2) equation 1 was solved with subprogram NNLS (Lawson & Hanson, 1974), which constrains solutions, x, to be positive. [b] Abbreviations: H_o, ordered α helix; H_d, disordered helix, correlated with the ends of helical segments, 3_{10} and α_{II} helix; S_a, antiparallel β strands; S_p, parellel β strands; T, turn as defined by Levitt & Greer (1977); U, undefined; H, total helix; B, total β strand. [c] The standard deviation was calculated from $S = ([\Sigma D_i^2 - (1/N)(\Sigma D_i)^2]/(N-1))^{1/2}$, where D_i = (Raman $\%_i$ − X-ray $\%_i$) for each of $N = 14$ proteins in the reference set and where each protein being analyzed is excluded from the reference set.

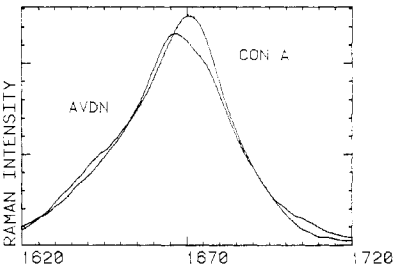

FIGURE 1: A comparison of amide I regions of avidin (maximum at 1667 cm^{-1}) and concanavalin A (maximum at 1672 cm^{-1}), showing the near coincidence of the two Raman bands.

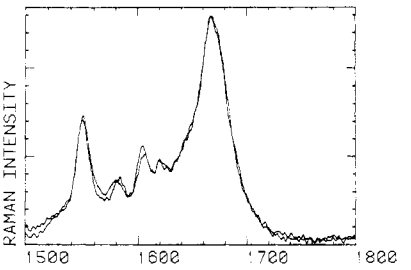

FIGURE 2: A comparison of Raman spectra of avidin crystals and avidin in solution. Differences in the spectra arise from subtle variations in background and, therefore, are not significant.

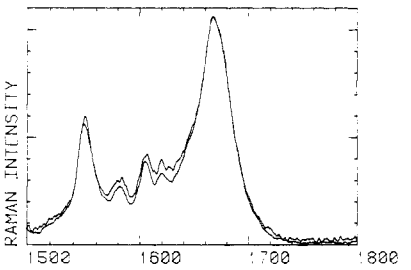

FIGURE 3: A comparison of Raman spectra of avidin and avidin–biotin in solution. As in Figure 2, differences here are not significant.

We relied on the work of Mwindaace et al. (1975) in the interpretation of the tyrosine doublet at 820–860 cm^{-1}. In a similar vein, our analysis of the conformation of the disulfide bridge of avidin is based on the work of Van Wart & Scheraga (1976a,b).

Programs were written to use the preference coefficients of Chou & Fasman (1974) and Levitt (1978) and the information coefficients of Robson (Garnier et al., 1978) in the prediction of secondary structure from the sequence of avidin. For the Robson algorithm, we used the decision constant appropriate for proteins possessing a high level of β structure.

Results and Discussion

The Raman spectra in the amide I region of avidin appears in Figure 1 compared with the corresponding spectrum of concanavalin A. The spectra of concanavalin A and avidin are relatively similar in the position and shape of their amide I bands. Quantitative analysis of the avidin spectrum as described under Materials and Methods bears out the qualitative similarities in the spectra of avidin and concanavalin A. Of the 14 proteins used as a basis set, concanavalin A has the highest correlation with avidin.

The estimates of secondary structure as well as the uncertainty in these estimates are given in Table I. Confidence intervals for these estimates can be calculated from the Student's t distribution with 13 degrees of freedom, since 14 independent observations of error have been made for 14 proteins (R. W. Williams, unpublished results). For example, the estimate of total helix is 10% with one standard deviation of 5%, indicating a 10% chance that the helix content is less than 3% and a 10% chance that it is greater than 17%. Our analysis indicates most of the helix may be disordered or in short segments. Disordered helical residues are defined here as the two residues on each end of a helical segment, 3_{10} and α_{II} helix; the elements of matrix F in eq 2 are constructed from known structures to reflect this distinction. The total β-strand content is 55% with a standard deviation of 4%. There is some evidence that a small fraction of the β structure may be in parallel strands. However, the average estimate for parallel strands (2.5 ± 4%) is not significant. Our quantitative estimates agree well with the qualitative findings of Green & Melamed (1966); the positive Cotton effect below 220 nm in their circular dichroism spectra indicated little or no α helix.

Amide I spectra of crystals of avidin in ammonium sulfate, avidin in water, and the complex of avidin with D-biotin in water are nearly identical in the region between 1630 and 1710 cm^{-1} (Figures 2 and 3). Furthermore, estimates of secondary structure (Table I) are identical within experimental error. These results are consistent with the identical sedimentation coefficient for the native protein and its biotin complex as well as the isomorphous diffraction of crystals of avidin and the avidin–biotin complex (Green & Joynson, 1970).

Avidin has but one tyrosine in each of its four identical subunits (De Lange & Huang, 1971). Thus, an analysis of the tyrosine doublet in the 820–860-cm^{-1} region will yield unambiguous information concerning the state of hydrogen bonding of the tyrosine (Mwindaace et al., 1975). The tyrosine

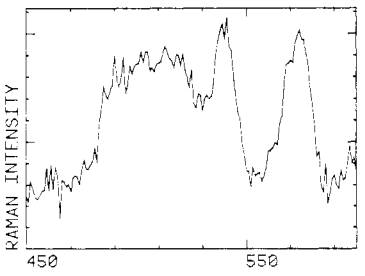

FIGURE 4: Region of disulfide stretching in Raman spectra (450–550 cm^{-1}). The band at 540 cm^{-1} is probably due to the disulfide of avidin; the source of the broad band at 510 cm^{-1} is not certain.

doublet of avidin appears at 826 and 855 cm^{-1} with an intensity ratio (826:855) of 1:2.35, close to the doublet position and intensity ratio of crystalline L-tyrosine hydrochloride (824 and 855 cm^{-1} and 1:2.5). Thus the environment of tyrosine-33 of avidin is similar to L-tyrosine hydrochloride, strong hydrogen bonding in which the phenolic oxygen serves primarily as an acceptor of a proton. An unprotonated tyrosine is an essential intermediate for the mechanistic pathway of nitration (Riordan & Vallee, 1972). The resistence of tyrosine-33 to modification by tetranitromethane even at pH 12.0 implies a buried residue, whose phenol oxygen does not acquire the phenoxide state. Results of the Raman work, then, are consistent with the low reactivity of tyrosine-33 to tetranitromethane.

The region of 500–550 cm^{-1} bears information concerning the lone disulfide of avidin subunits. On the basis of model studies of Van Wart & Scheraga (1976a,b), disulfide bonds with dihedral angles of 85 ± 20° absorb between 505 and 550 cm^{-1} due to a stretching mode. Disulfide bonds under conformational strain (dihedral angles significantly different from 85 ± 20°), however, absorb between 450 and 500 cm^{-1}. The absence of active Raman modes from 450 to 500 cm^{-1} for avidin (Figure 4) is evidence for a disulfide bond with a dihedral angle of 85 ± 20°. The dihedral angle for the avidin disulfide, then, falls in the relatively narrow range of dihedral angles observed in the majority of protein structures (Richardson, 1981).

Provided the S–S link is not under strain, the frequency of the stretching mode is sensitive to the conformation of each of the two dihedral angles C_β–S. The sharp resonance at 540 cm^{-1} of avidin corresponds to a stretching mode of a disulfide whose dihedral angles C_β–S are 0–50°. In addition to the resonance at 540 cm^{-1}, however, there may be a broad and relatively weak band centered at 510 cm^{-1}. A disulfide stretch at 510 cm^{-1} correspnds to dihedral angles of C_β–S of 50–180°. Although assignment of the Raman modes in the region of 510 cm^{-1} is uncertain, the presence of an additional peak in the disulfide stretching region hints at the possibility of a mixture of conformations for the disulfide of avidin. Disorder of disulfide links has been observed in the Bence–Jones V_L dimer REI (Epp et al., 1975), where disulfides exist in both left- and right-handed conformations.

The conformational preference values of Levitt (1978), Chou & Fasman (1974), and Robson (Garnier et al., 1978) for the prediction of secondary from the primary structure of proteins yield five regions of strong β preference. In Table II we list these regions of predicted β structure, as well as the entire fraction of residues in an extended conformation. In addition to these β regions, the Robson method with an unbiased decision constant and the Chou and Fasman and Levitt preference values indicate helix formation for residues 89–102, a region that also has a high preference for an extended conformation. A decision constant in the Robson algorithm to reflect the high β content of avidin supresses the helical

Table II: Predicted Secondary Structure for Avidin

method	β regions	α regions	% β	% α
Chou–Fasman[a]	4–8, 16–24, 29–41, 63–72, 75–85, 102–106, 111–127	89–101	55	10
Levitt[a]	16–24, 28–41, 52–56, 62–72, 74–85, 102–106, 111–124	89–101	55	10
Robson[b] (β biased)	1–6, 8–10, 17–23, 30–38, 63–68, 79–85, 92–100, 104–106, 110–122		49.2	0.0

[a] We used an average preference based on five residues in succession to predict a β strand and an average preference based on six residues to predict helix. If the cumulative preference for β strand was greater than or equal to 1.05, we assigned β structure to that region. With a value of 1.05, the percentage of residues predicted in β strand agrees with the Raman estimate of 55%. Similarly, we chose the cut-off preference for helix so as to place 13 residues (10% of the total) in the helix category. The minimum preference for helix was 1.12. [b] We required three adjacent residues of extended conformation in the Robson method in order to include the residues under the β classification. An additional four single residues, however, belonged to the extended category, bringing the total percentage of extended residues to 52.3.

preference of residues 89–102 in favor of β conformation. Conformational predictions for the first 12 residues of the amino terminus and residues 39–56 lack consistency among the algorithms. Residues of these regions are predicted as a mixture of turn, coil, and β conformations.

Since a confidence interval can be calculated for the Raman estimate of β-strand content, it is possible to evaluate the accuracy of the prediction made by the Robson sequence algorithm. The sequence prediction is 49% β strand. Our analysis of the Raman data indicates a 10% probability of a β-strand content less than 50%.

On the basis of our analysis of the amide I band and the prediction algorithms, we suggest the subunit of avidin has at least five strands of β structure. Thus, avidin may be similar to the class of β sandwich proteins characteristic of the domains of the immunoglobulins (Epp et al., 1975) and human prealbumin (Blake et al., 1978). In fact, the subunit size (14000 daltons), percentage of residues in α (9%) and β (45%) conformations, and the physical properties of prealbumin are similar to those of avidin. We detect no sequence homology between the two proteins. Nonetheless, a structural variation of the hydrophobic core found in β sandwich structures such as human prealbumin may provide an ideal hydrophobic pocket for the binding of biotin. Crystallographic investigations underway in other laboratories will lead hopefully to a well-determined tertiary structure for avidin and a clear understanding of the biotin–avidin interaction.

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Nanosecond Rotational Motions of Apolipoprotein C-I in Solution and in Complexes with Dimyristoylphosphatidylcholine†

Ana Jonas,* Jean-Paul Privat, Philippe Wahl, and James C. Osborne, Jr.

ABSTRACT: Human apolipoprotein C-I (apo C-I) in solution, in monomeric and oligomeric form, and in micellar complexes with dimyristoylphosphatidylcholine (DMPC), below and above the phase transition temperature of DMPC, was investigated with steady-state and time-resolved fluorescence methods. The environment of the Trp residue of apo C-I, in each physical state, was evaluated from fluorescence spectra and their changes upon KI quenching. Rotational correlation times of Trp residues were obtained from fluorescence anisotropy decay measurements. Static fluorescence anisotropy was determined as a function of temperature for the Trp residues of apo C-I in all physical states and for diphenylhexatriene dissolved in apo C-I·DMPC complexes. It was found that the Trp residues of apo C-I in solution are exposed from 75 to 88% to the aqueous medium, depending on the state of self-association. On the other hand, the Trp residues in apo C-I·DMPC complexes are only 42–45% exposed to KI quenching through an environment distinct from water. Apolipoprotein C-I in all its physical forms had two rotational correlation times associated with Trp motions: a longer one dependent on the size and flexibility of the entire particle and a very short one in the range from 0.2 to 0.4 ns. The later correlation times correspond to local Trp residue motions. These Trp motions were not significantly affected by a transition from the gel to the liquid-crystalline state of the lipid in apo C-I·DMPC complexes, suggesting that there is no coupling between the local motions of lipids and those of Trp side chains of apo C-I.

The soluble apolipoproteins of the A and C classes, isolated from plasma lipoproteins, have variable amounts of α-helical structure in aqueous solutions. Their content of secondary structure usually increases upon self-association to various oligomeric forms, which depend on the nature and the concentration of the apolipoproteins (Osborne & Brewer, 1977; Morrisett et al., 1977). Human apo A-I, the major apolipoprotein of high density lipoproteins (HDL),[1] exists as an elongated monomer or as very asymmetrical oligomers in aqueous buffers (Barbeau et al., 1979) and exhibits a low free energy of denaturation—2.4 kcal/mol as compared to 7 kcal/mol for other proteins (Tall et al., 1976). Upon lipid binding, the α-helix content of the A and C apolipoproteins increases further, up to 70–80%, which is in the range of α-helical structure of the apolipoproteins in native HDL particles (Lux et al., 1972; Morrisett et al., 1977). The ability of these apolipoproteins to change dramatically their secondary structure, to denature very easily, and to exist in monomeric and oligomeric states in water as well as in micellar or vesicular complexes with phosphatidylcholines (Jonas et al., 1980; Patterson & Jonas, 1980b) or in spherical native lipoproteins suggests a remarkable degree of structural adaptability. This concept of structural adaptability of apolipoproteins is further supported by the observation that one type of apolipoprotein can exchange for another in native or synthetic lipoproteins, without changing significantly the overall properties of the particles (Lagocki & Scanu, 1980; Rosseneu et al., 1981).

† From the Department of Biochemistry and School of Basic Medical Sciences, University of Illinois, Urbana, Illinois 61801 (A.J.), Centre de Biophysique Moléculaire, CNRS, 45045 Orléans Cedex, France (J.-P.P. and P.W.), and Molecular Disease Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205 (J.C.O.). *Received June 15, 1982.* This work was carried out at the Centre de Biophysique Moléculaire, CNRS, Orléans, France, and was supported in part by a Fogarty Fellowship of the NIH–French CNRS Program for Scientific Collaboration and National Institutes of Health Grant (HL-16059) to A.J.

[1] Abbreviations: HDL, high-density plasma lipoproteins; apo A-I, major protein component of HDL; apo C-I, minor protein component of HDL and a relatively more important component of very low density lipoproteins; DMPC, dimyristoylphosphatidylcholine; apo C, apolipoproteins of the C class, which include human apo C-I, and animal apolipoproteins of low molecular weight isolated from HDL or from very low density lipoproteins; DPH, 1,6-diphenyl-1,3,5-hexatriene; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride.

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