Purification and Molecular Properties of Rabbit Lung Indolamine *N*-Methyltransferase†

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ABSTRACT: Indolamine *N*-methyltransferase (INMT) has been purified to an apparent homogeneity from rabbit lung, and some of its catalytic and physicochemical properties have been examined. The enzyme is a monomeric protein with a molecular weight of 31 500 ± 1000, a molecular Stokes radius of 21.5 Å, and a diffusion coefficient of 8.7×10^{-7} cm^2/s. The frictional ratio of the native enzyme (1.05) suggests that the shape of the molecule is nearly spherical. Denaturation experiments performed with increasing concentrations of guanidine hydrochloride (Gdn·HCl) at neutral pH indicated that the active site of the enzyme was destroyed by a structural rearrangement of the protein molecule without large change in its size and shape. The final state reached in 6.0 M Gdn·HCl seemed to correspond to a disulfide cross-linked randomly coiled polypeptide. Full normalization of the fluorescent parameter was attained only in the presence of 0.1 M β-mercaptoethanol. A structural rearrangement has been observed upon acidification of INMT from pH 7.0 to pH 2.0. At pH 4.5, most of the peptide backbone appeared to be unorganized, but further acidification to pH 2.0 produced a reorganization of protein structure which became able to bind 8-anilino-1-naphthalenesulfonate. The data support the hypothesis that the enzyme structure results from the close package of organized regions joined by structureless segments.

Indolamine *N*-methyltransferase (INMT),[1] an enzyme first discovered by Axelrod (1962), transfers the methyl group from SAM to the amino group of a variety of indolamines. The enzyme has a broad substrate specificity, being able to N-methylate other aromatic biogenic amines as well as various foreign compounds and drugs (Narasimhachari & Lin, 1974, 1976). It appears to act with the reaction mechanism "ordered Bi-Bi" with SAM as the first substrate bound (Lin et al., 1973; Porta et al., 1977a). The enzyme is highly localized in the cytosol fraction of rabbit lung although it has been reported that other mammalian tissues can also enzymatically convert indolamines in their N-methylated derivatives (Mandell & Morgan, 1971; Mandel et al., 1972; Narasimhachari et al., 1972; Wyatt et al., 1973; Bhikharidas et al., 1975).

The functional significance of INMT is unknown at present. In fact, the oxidative deamination appears to be the major pathway for the indolamine inactivation, and on the other hand, the specific action of N-methylated indolamines cannot be excluded (Sellinger et al., 1978). Moreover, since it has been shown that *N*-methyltryptamine is the indolamine substrate with the lowest K_m (Mandel et al., 1971), it is possible that the physiological role of the enzyme may be the formation of *N*,*N*-dimethyltryptamine (DMT). However, since DMT has been recognized to be a potent psychotogenic in man, its production in substantial quantities could cause mental aberrations. In this respect, as reviewed elsewhere (Porta et al., 1979a), an abundant literature exists about a possible involvement of INMT activity in the etiology of some psychotic disorders.

In order to elucidate the relationship between the structure of INMT and its kinetic and biological characteristics, we have undertaken the characterization of its molecular properties. In this paper, we describe a method of obtaining a substantial amount of homogeneous enzyme and some of its basic catalytic and physicochemical properties.

Materials and Methods

Materials. Commercial sources of reagents and materials were as follows: tryptamine hydrochloride, *N*-methyltryptamine, reduced DTT, $NaDodSO_4$, MSH, and sperm whale myoglobin (Myo) were supplied by Sigma Chemical Co., St. Louis, MO; DEAE-cellulose (DE-52) was from Whatman, Maidstone, U.K.; hydroxylapatite (Bio-Gel HT) was from Bio-Rad Laboratories, Richmond, CA; Sephadex G-75, Sepharose 4B, AH-Sepharose 4B, and Sepharose 6B were from Pharmacia Fine Chemicals, Uppsala, Sweden; cytochrome *c*, chymotrypsinogen A (CHGA), ovalbumin (Ova), and bovine serum albumin (BSA) were from Boehringer-Mannheim, West Germany; acrylamide and methylenebis(acrylamide) were from Fluka, Buchs, Switzerland; *N*,*N*,*N*′,*N*′-tetramethylethylenediamine was from Eastman Organic Chemicals, Rochester, NY. *S*-Adenosylmethionine was prepared as described by Pegg & Williams-Ashman (1969); *S*-[*methyl*-^{14}C]adenosylmethionine (specific activity 60 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, Bucks, U.K. The Hg-coupled Sepharose was prepared by reaction of AH-Sepharose 4B with *p*-(chloromercuri)benzenesulfonyl chloride as previously described (Porta et al., 1977b). All other chemicals were the purest available grades from standard commercial sources.

Enzyme Assay. The INMT assay and the analysis of reaction products were performed as previously described (Porta et al., 1977a, 1979b). One unit of enzyme activity was defined as the amount which produced 1 nmol of product per h at 37 °C. Specific activity was expressed as the number of units per milligram of protein. The N-methylation of tryptamine was linear with time and with the amount of enzyme used under the experimental conditions. Protein concentration was determined according to the method of Lowry et al. (1951), with bovine serum albumin as a standard, for crude and partially purified enzyme preparations. The protein concentration of purified enzyme preparations was determined as stated above and/or spectrophotometrically at 210 and 205 nm (Gratzer, 1976; Alexander et al., 1978).

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[1] Abbreviations: INMT, indolamine *N*-methyltransferase; SAM, *S*-adenosylmethionine; DMT, *N*,*N*-dimethyltryptamine; DTT, dithiothreitol; MSH, β-mercaptoethanol; BSA, bovine serum albumin; Ova, ovalbumin; CHG, chymotrypsinogen; Myo, myoglobin; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism; Gdn·HCl, guanidine hydrochloride; ANS, 8-anilino-1-naphthalenesulfonate.

0006-2960/82/0421-1464$01.25/0

Table I: Purification of Indolamine *N*-Methyltransferase

step	volume (mL)	total protein (mg)	total activity (units)	sp act. (units/mg)	yield (%)	*x*-fold purification
$(NH_4)_2SO_4$, 0–80%	1450	62250	54810	0.84	100	1.0
DEAE-cellulose	75	2438	23259	9.54	42.4	11.4
Hg-coupled Sepharose	3.5	490	10261	20.94	18.7	24.9
Sephadex G-75	25	66.8	8684	130	15.8	154.8
hydroxylapatite I	40	28.9	8526	295	15.6	351.2
hydroxylapatite II	20	25.5	8415	330	15.4	392.9

Polyacrylamide Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed at pH 8.6 in Tris–glycine buffer (6 g of Tris, 28.8 g of glycine, water to 1.0 L). All gels (7.5% polyacrylamide) were prepared according to the method of Davis (1964).

Electrophoresis on a polyacrylamide gel (7.5 or 10% polyacrylamide) containing 0.1% $NaDodSO_4$ and 0.1% MSH was carried out according to the method of Weber & Osborn (1969). The purified INMT and the marker proteins were pretreated at 37 °C for 2 h with 0.1% $NaDodSO_4$ containing 0.1% MSH before the electrophoretic run.

The samples (10–50 μg each) were subjected to electrophoresis at a constant current of 2 mA/tube. Gels were stained with Coomassie Brilliant Blue (0.1% in 5% methanol/7.5% acetic acid) for 15 h at 20 °C and destained with frequent changes of acetic acid/methanol/water (3:2:35 v/v).

Analytical Gel Chromatography. Molecular weight, Stokes radius, and diffusion coefficient determinations of rabbit lung INMT were carried out as described by Andrews (1964, 1970) and Laurent & Killander (1964) on a Sephadex G-75 column (1.5 cm × 90 cm) previously equilibrated in 25 mM potassium phosphate buffer, pH 7.3, containing 1.0 mM DTT and 0.15 M KCl. The calibrating proteins were detected in 1.0-mL fractions eluted at a flow rate of 3.0 mL/h by their extinction at 280 nm. INMT was detected by enzymic assay. The parameters for standard proteins, taken from the literature and used for the calculations of molecular weight (M_r), Stokes radius (a), and diffusion coefficient ($D_{20,w}$) values, were the following: sperm whale myoglobin, M_r 17 000; a, 18.8 Å; $D_{20,w}$, 10.3; chymotrypsinogen A, M_r 25 000; a, 22.4 Å; $D_{20,w}$, 9.5; ovalbumin, M_r 45 000; a, 27.3 Å; $D_{20,w}$, 7.8 (Ackers, 1964; Laurent & Killander, 1964; Siegel & Monty, 1966; Andrews, 1970; Sober, 1970).

The behavior of the purified INMT on molecular-sieve chromatography was also examined by a Sepharose 6B column (1.0 cm × 60 cm), equilibrated in 25 mM potassium phosphate buffer, pH 7.0, containing 6.0 M Gdn·HCl and 0.1 M MSH, in order to observe possible heterogeneity and/or subunit structure. The samples (purified INMT and calibrating proteins) were pretreated at 37 °C for 60 min with 6.0 M Gdn·HCl and 0.1 M MSH and then singly chromatographed (0.1 mL each). The enzyme and the marker proteins eluted in 0.5-mL fractions at a constant flow rate of 1.5 mL/h were detected by the fluorescence emission at 340 nm.

Ultraviolet Fluorescence. Fluorescence spectra and intensities were obtained with a Perkin-Elmer MPF-2A spectrofluorometer. Fluorescence measurements were made in the range where emission was linear with protein concentration. The absorbance of all solutions was less than 0.1 at the excitation wavelength. The temperature of the solution cell was maintained at 22 °C. Simultaneous readings were made on control solutions to correct for changes in the lamp intensity or for fluctuations in the photomultiplier response.

Fluorescence Polarization. Polarization measurements were performed on a Perkin-Elmer MPF-2A spectrofluorometer. The polarization was calculated from $P = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$ where $G = I_{hv}/I_{hh}$, I is the intensity, and the first and the second subscripts refer to the plane of the excitation and emission beams (v, vertical; h, horizontal).

Circular Dichroism. A Cary Model 61 CD recording spectropolarimeter, equipped with a CD difference accessory, was used to record the CD directly in terms of ellipticity (θ). A mean residue weight of 115 was used for the peptide chromophore. Cells of 0.2 cm were used for measurements in the far-ultraviolet by using protein solutions, the absorbance of which was between 0.4 and 0.7 at 280 nm in a 1-cm cell. The solvent contribution to the CD spectra was subtracted either by using the instrument in the difference mode, with the solvent in the reference compartment, or by obtaining a spectral curve on the solvent directly. The spectra reported in this paper represent the average of three or more tracings.

Ultraviolet Absorption. Normal and second-derivative spectra were recorded with a Perkin-Elmer Model 575 spectrophotometer equipped with an electron derivative accessory (Hitachi 200-0507 derivative spectrum unit). A Zeiss PMQ II was used for absorption measurements at specific wavelengths.

Results

Enzyme Purification. The purification procedure, summarized in Table I, includes some initial steps (extraction, ammonium sulfate precipitation, and DEAE-cellulose chromatography) essentially performed as previously described for a partial purification of INMT (Porta et al., 1979c). The fractions from the DEAE-cellulose column were pooled, dialyzed against the extraction buffer, and freeze-dried. The dry powder was redissolved with 10 mM potassium phosphate buffer, pH 7.3, containing 5 mM DTT in order to reduce quantitatively the protein SH groups. The resulting solution was dialyzed overnight against the same buffer. The dialyzed sample was filtered through a Sephadex G-25 column (2.5 cm × 20 cm), previously equilibrated with 10 mM potassium phosphate buffer, pH 7.3, to remove DTT and then immediately passed through a Sepharose mercurial column (3.0 cm × 14 cm) equilibrated with 0.2 M potassium phosphate buffer, pH 7.3. The elution was performed stepwise with (a) equilibrating buffer containing 2.0 M KCl (120 mL) and (b) equilibrating buffer containing 2.0 M KCl and 10 mM DTT. Fractions of 8 mL were collected at a flow rate of 0.5 mL/min. The elution pattern showed two peaks of INMT activity which were pooled singly. The first pool, eluted with buffer a above and containing the majority of enzyme activity, was dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.3, and 1.0 mM DTT and then freeze-dried. The dry powder, redissolved with small volume of 10 mM potassium phosphate buffer, pH 7.3, and 1.0 mM DTT, was subjected to chromatography on a column of Sephadex G-75 (2.0 cm × 130 cm) equilibrated with 25 mM potassium phosphate buffer, pH 7.3, and 1.0 mM DTT. The enzyme, eluted at a flow rate of 6.0 mL/h, appeared in a single peak. The most active fractions were pooled and applied onto a hydroxylapatite column (4.0 cm × 4.0 cm) equilibrated with 25 mM potassium phosphate

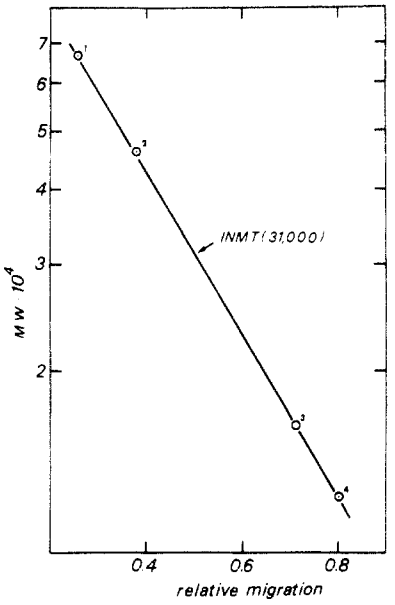

FIGURE 1: Molecular weight determination of purified rabbit lung INMT by $NaDodSO_4$–polyacrylamide gel electrophoresis. The electrophoresis was carried out as described in the text. (1) BSA; (2) Ova; (3) Myo; (4) cytochrome *c*.

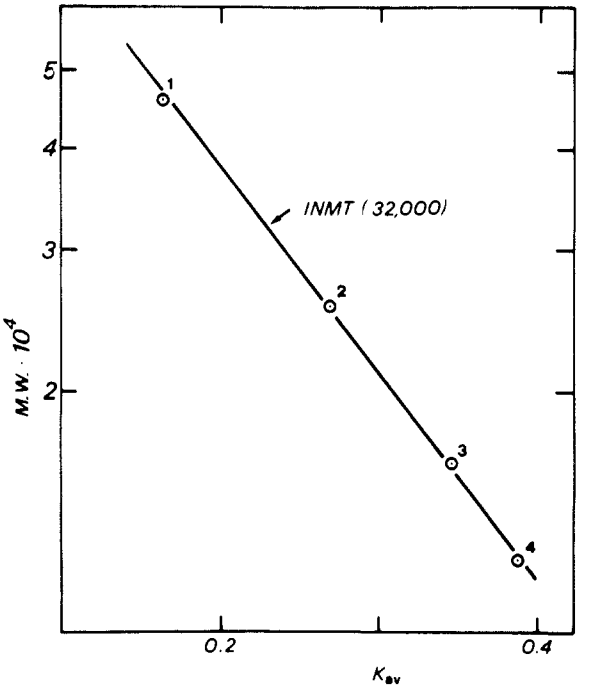

FIGURE 2: Molecular weight determination of purified rabbit lung INMT by Sepharose 6B gel filtration in 6.0 M Gdn·HCl and 0.1 M MSH, pH 7.0. (1) Ova; (2) CHG; (3) Myo; (4) cytochrome *c*. Other experimental details are described in the text.

buffer, pH 7.3, and 1.0 mM DTT. Fractions of 3.0 mL were collected at a flow rate of 0.3 mL/min from a linear gradient (200 mL) of KCl (0.025–0.2 M) in the same buffer.

The final step of the purification was a rerun, after dialysis of the pooled active fractions, through the same hydroxylapatite column under identical conditions. In fact, some attempts at further purification of the enzyme resulted only in loss of its activity.

Criteria of Purity and Stability. The homogeneity of the final active peak of INMT was demonstrated by using polyacrylamide gel electrophoresis in both the presence and absence of $NaDodSO_4$ as described under Materials and Methods. A single protein band was always observed. Further evidence of protein homogeneity was provided by gel filtration on Sepharose 6B equilibrated in 6.0 M Gdn·HCl/0.1 M MSH. The elution of a single symmetrical peak confirmed the essential enzyme homogeneity.

The highly purified INMT preparation required sulfhydryl-protecting agents, such as DTT or MSH, for optimal stability. Thus, the enzyme could be stored at −50 °C in the presence of 5 mM dithiothreitol for at least 2 months with minimal loss of activity. The INMT protection by sulfhydryl agents was also observed during the course of purification, since higher recoveries of enzyme activity from chromatographic columns were obtained when DTT was added to the eluant buffers. Finally, the addition of BSA to the incubation mixture had a protecting effect too, probably against enzyme inactivation in protein-diluted solutions (0.05 mg/mL).

Kinetic Properties. The kinetical analysis of the initial velocity of the purified INMT showed that the determined apparent K_m values for SAM (0.5×10^{-4} M) and tryptamine (0.7×10^{-3} M) were similar to the ones previously obtained with partially purified enzyme (Mandel et al., 1971; Wyatt et al., 1973; Borchardt, 1974; Porta et al., 1977a). Moreover, the optimal pH of 7.9 was also confirmed after assays in 0.1 M potassium phosphate buffer between pH values of 6.0 and 9.0. It is noteworthy that all the kinetic studies were performed in the presence of 1.0 mM DTT. In fact, the presence in the incubation mixture of DTT increases the enzyme activity about 100%, suggesting an involvement of thiol groups in the catalytic process. This finding confirms previous data (Porta et al., 1979c) showing a strong inhibition of the enzyme by *p*-(chloromercuri)benzoic acid.

Molecular Weight. (a) Polyacrylamide Gels. The mobility of rabbit lung INMT in 0.1% $NaDodSO_4$ and 0.1 M MSH on 7.5% polyacrylamide gels was measured and compared with that of proteins of known molecular weight. Figure 1 shows a plot of the logarithm of the molecular weight of the standard proteins against their mobility. The molecular weight of INMT was estimated to be 31 000. A similar result was obtained by gel electrophoresis on 10% polyacrylamide, 0.1% $NaDodSO_4$, and 0.1 M MSH (data not shown).

(b) Gel Chromatography. The molecular weight of purified rabbit lung INMT was also estimated in a calibrated Sephadex G-75 column equilibrated in 0.025 M potassium phosphate buffer, pH 7.3, containing 1.0 mM DTT and 0.15 M KCl. The result (31 000) was consistent with that obtained by polyacrylamide gel electrophoresis.

Gel chromatography on Sepharose 6B equilibrated with 6.0 M Gdn·HCl and 0.1 M MSH yielded a single peak with an estimated molecular weight of 32 000 (Figure 2). Therefore, the results obtained with different techniques indicate that rabbit lung INMT is a monomeric protein having a molecular weight of 31 500 ± 1000.

Molecular Stokes Radius, Diffusion Coefficient, and Frictional Ratio. The Stokes radius of rabbit lung INMT was determined from gel filtration data according to Laurent & Killander (1964) with ovalbumin, α-chymotrypsinogen, and myoglobin as reference proteins. The distribution coefficient (K_{av}) of each reference protein was determined on a Sephadex G-75 column developed as described under Materials and Methods. Figure 3 shows the linear correlation obtained by plotting $(-\log K_{av})^{1/2}$ against the Stokes radius. From this plot, the Stokes radius of INMT was determined to be 21.5 Å.

For measurement of the asymmetry of the molecule, the frictional coefficient (f/f_0) was calculated with the following equation (Siegel & Monty, 1966):

$$f/f_0 = a/[3\bar{v}M/(4\pi N)]^{1/3}$$

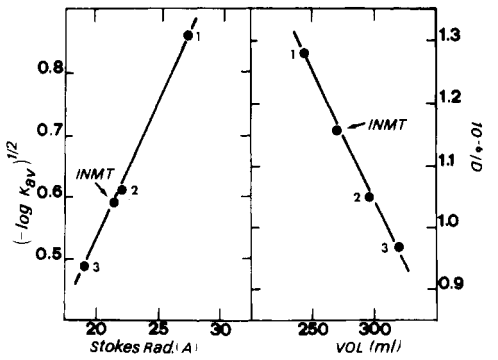

FIGURE 3: Molecular Stokes radius (left panel) and diffusion coefficient (right panel) of rabbit lung INMT determined by Sephadex G-75 gel chromatography in 0.025 M potassium phosphate buffer, pH 7.3, containing 1.0 mM DTT and 0.15 M KCl. (1) Ova; (2) CHG; (3) Myo. Other experimental details are described in the text.

Table II: Molecular Parameters of INMT

parameter	value
mol wt	
by gel filtration in potassium phosphate	31000
by gel filtration in 6.0 M Gdn·HCl/0.1 M MSH	32000
by $NaDodSO_4$ gel electrophoresis	31000
$D_{20,w} \times 10^{-7}$ (cm^2/s)	8.7
Stokes radius (Å)	21.5
frictional coefficient, f/f_0	1.05

where a is the Stokes radius, $\bar{v}$ is the partial specific volume, M is the molecular weight, and N is Avogadro's number. A partial specific volume of 0.725 was assumed in the calculation. The f/f_0 value of rabbit lung INMT was found to be 1.05.

The value of the diffusion coefficient ($D_{20,w}$) of INMT, determined by using the same gel filtration data according to the method of Andrews (1964), was determined to be 8.7×10^{-7} cm^2/s. The molecular parameters of rabbit lung INMT are summarized in Table II.

Aromatic Amino Acid Composition. The tryptophan and phenylalanine contents of INMT were determined by second-derivative spectroscopy as described by Balestrieri et al. (1978). The results were 14 and 6 mol of residue per mol of protein for phenylalanine and tryptophan, respectively. The number of tyrosyl residues was determined by the increase of the absorbance at 295 nm upon alkalinization by using the extinction coefficient reported by Donovan (1969) ($\Delta\epsilon$ = 2480); 18 mol of tyrosine per mol of INMT was found by this techinque.

Effect of Acid. (*a*) *Protein Fluorescence.* The fluorescence emission maximum of rabbit lung INMT resulting from 295-nm excitation is centered at 338 nm, which is typical of native proteins containing tryptophanyl residues partially in contact with the solvent (Chen et al., 1969). In fact, the fluorescence emission of buried tryptophan in proteins occurs at shorter wavelengths (320–330 nm) than tryptophan in aqueous solution (350–360 nm) (Teale, 1960). The acid pH dependence of tryptophanyl fluorescence intensity at 340 nm of rabbit lung INMT is reported in Figure 4. Between pH 9.0 and pH 6.0, the emission intensity as well as the fluorescence maximum was unchanged. Lowering the pH from 6.0 to 4.5 resulted in a steep decrease of the emission intensity at 340 nm without a shift in the emission maximum. Further acidification to pH 2.0 produced a monotonic decrease of the fluorescence intensity at 340 nm but did not change the emission maximum, which remained still centered at 338 nm. Back-titration from pH 2.0 to 7.0 did not produce recovery of the initial fluorescence intensity at 340 nm.

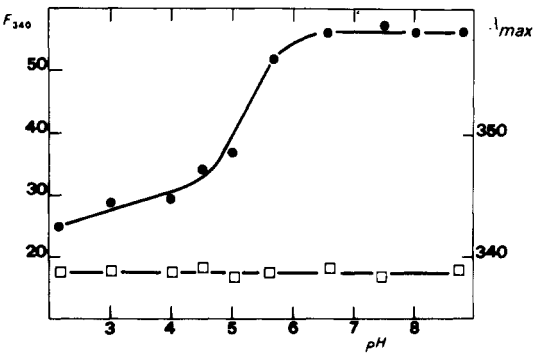

FIGURE 4: Acid pH dependence of the tryptophanyl fluorescence emission at 340 nm (●) and the emission maximum (□) of rabbit lung INMT in 0.05 M potassium phosphate, 0.01 M potassium acetate, and 0.15 M KCl. Excitation was at 295 nm. Protein samples were prepared by diluting a 1:20 concentrated solution of INMT (optical density at 280 nm = 1.0).

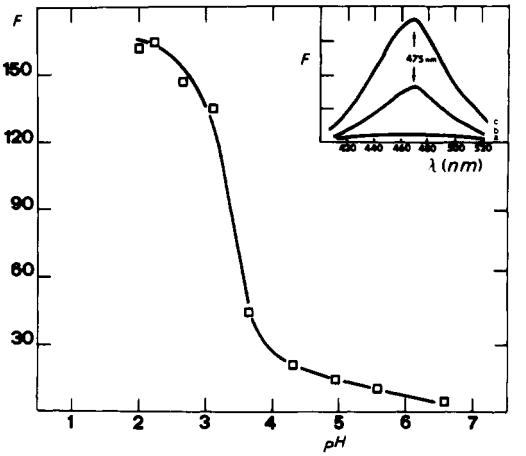

FIGURE 5: Acid pH dependence of the ANS fluorescence emission at 480 nm (□) in the presence of INMT. Protein solutions were prepared as described in Figure 4. The molar ratio between ANS and INMT was 10:1. Excitation was at 360 nm. Solvent was 0.05 M phosphate, 0.01 M acetate, and 0.15 M KCl. Inset: Emission spectra of the ANS/INMT conjugate: (a) pH 6.5; (b) pH 3.5; (c) pH 2.6.

(*b*) *ANS Fluorescence.* It is well-known that the ANS fluorophore is virtually nonfluorescent in aqueous solution but becomes fluorescent in organic solvents or when bound to certain proteins and enzymes (Stryer, 1965; Weber & Daniel, 1966; Turner & Brand, 1968). The fluorescence properties of ANS are not modified by INMT at neutral pH, thus indicating that the molecular structure of the native enzyme does not contain any binding site for the extrinsic chromophore. On the other hand, the fluorescence intensity of ANS is strongly enhanced and its emission peak shifted from 530 to 475 nm when it reacts with INMT at pH 3.0. The acid pH dependence of ANS fluorescence in the presence of INMT is shown in Figure 5. The fluorescence of the extrinsic chromophore is slightly increased between pH 7.0 and pH 4.5, i.e., in the same pH range where most of the tryptophanyl fluorescence is reduced. The converse holds for the pH range from 4.5 to 3.0: in fact, in this pH range, the ANS fluorescence is strongly enhanced whereas the tryptophanyl fluorescence is monotonically reduced.

(*c*) *Circular Dichroism.* The CD activity of INMT in the far-ultraviolet provides information about its secondary structure. Figure 6 shows the far-ultraviolet CD spectra from 240 to 210 nm obtained at three different pH values, i.e., 7.3, 3.5, and 2.0. At neutral pH, the CD spectrum of the native enzyme contains a negative extremum at 222 nm typical of

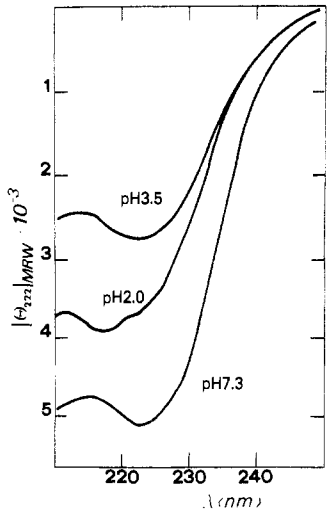

FIGURE 6: Far-ultraviolet CD spectra of rabbit lung INMT in 0.05 M phosphate, 0.01 M acetate, and 0.15 M KCl at pH 7.3 (lower curve), pH 3.5 (upper curve), and pH 2.0 (middle curve). Protein absorbance OD^{1cm}_{280nm} = 0.4. Cell path length = 0.2 cm.

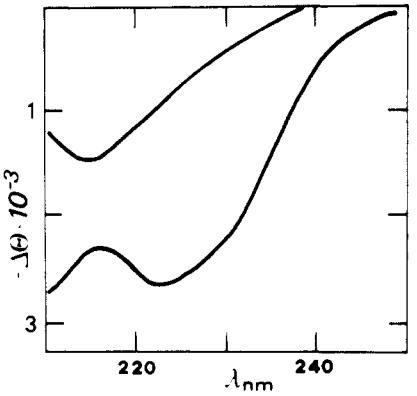

FIGURE 7: Far-ultraviolet CD difference spectra of rabbit lung INMT. The experimental conditions are those described in Figure 6. Lower curve, pH 3.5 vs. pH 7.3; upper curve, pH 2.0 vs. pH 3.5.

polypeptides in the α-helical conformation (Chen et al., 1974). Lowering the pH from 7.3 to 3.5 produced a decrease of the negative ellipticity. Further acidification to pH 2.0 resulted in an unexpected increase of the negative ellipticity, even though the CD spectrum at pH 2.0 showed features which differ from those contained in the CD spectrum at neutral pH. In fact, the CD spectrum at pH 2.0 shows a negative extremum at 216 nm and a shoulder at 222 nm. The amount of α-helical structure was determined at 224 nm (Richards, 1974, 1977) and resulted in 18%, 10%, and 14% at pH 7.3, 3.5, and 2.5, respectively. No further analysis of the CD data was carried out.

The nature of the structural changes occurring as the pH is lowered has been qualitatively estimated by examining the CD difference spectra between pH 7.3 and 3.5 and between pH 2.0 and 3.5 (Figure 7). The CD difference spectrum generated by lowering the pH from 7.3 to 3.5 shows only the negative extremum at 222 nm, thus indicating that the molecular structure of INMT loses a certain amount of helical structure. Further decrease to pH 2.0 generates a difference CD spectrum with a minimum at 216 nm. Since the intensity of CD activity at pH 2.0 is higher than that obtained at pH 3.5, it is reasonable to admit that the change reflects an increase of β structure. Within the approximations of our evaluation, it appears that the increase of β structure depends on the reorganization of random groups since the content of α-helical structure also increased from pH 3.5 to 2.0.

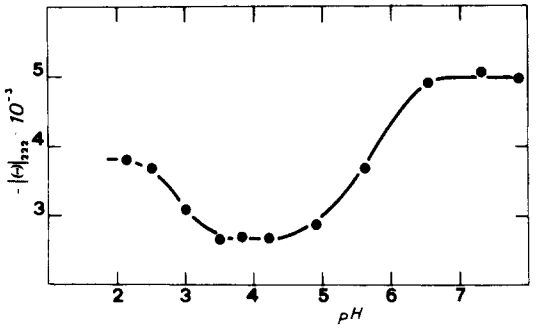

FIGURE 8: Acid dependence of the mean residue ellipticity at 222 nm of rabbit lung INMT. The experimental conditions are described in Figure 6.

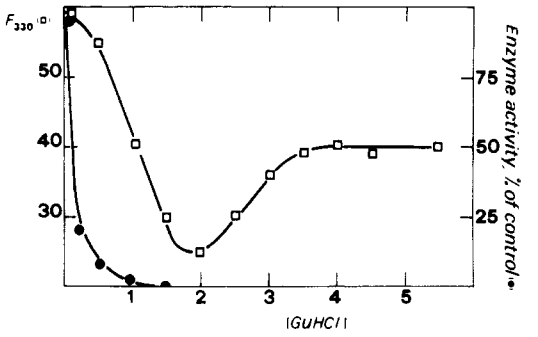

FIGURE 9: Effect of Gdn·HCl concentration on the tryptophanyl fluorescence emission (□) and the enzyme activity (●) of rabbit lung INMT at pH 7.9. Protein samples for fluorescence measurements were prepared as described in Figure 4. Excitation was at 295 nm. Enzyme assays were performed as described under Materials and Methods.

The acid pH dependence of the mean residue ellipticity at 222 nm is shown in Figure 8. There is no change in the CD activity at 222 nm until pH 6.5, after which a sharp decrease is apparent. The midpoint of the acid denaturation obtained following the CD activity at 222 nm occurs at about pH 5.5, which is in good agreement with the value obtained following the acid pH dependence of the tryptophanyl fluorescence at 340 nm. No further change of CD activity occurs between pH 4.5 and 3.5 while the increase of ellipticity observed as the pH is lowered from 3.5 to 2.0 reflects the same molecular phenomenon which leads to the increase of ANS fluorescence at strongly acid pH values (Figure 5). The ellipticity values as well as the shape of the CD spectrum at pH 3.5 and at pH 2.0 (Figure 6) indicate that the protein is predominantly in unordered form, but centers of organized peptide structure are still present in the acid-denatured form(s) of INMT.

Back-titration from pH 2.0 to pH 7.3 produced a CD spectrum which was similar to that obtained at pH 3.5. Therefore, it appears that the limited degree of unfolding which takes place between pH 7.3 and 3.5 is not reversible.

Effect of Guanidine Hydrochloride Concentration. (*a*) *Protein Fluorescence.* The effect of increasing the concentration of Gdn·HCl on the tryptophanyl fluorescence of rabbit lung INMT at pH 7.3 is shown in Figure 9. The emission intensity change at 330 nm is rather complex; between 0 and 2.0 M Gdn·HCl, it decreases almost linearly although no shift in the emission peak occurs in the same range of denaturant concentrations (Figure 10). A further increase of Gdn·HCl concentration from 2.0 to 4.0 M produces an enhancement of the tryptophanyl emission at 330 nm and a concomitant red shift of the emission maximum from 338 to 345 nm (Figures 9 and 10). No further change is observed by increasing the denaturant concentration from 4.0 to 6.0 M. At the latter

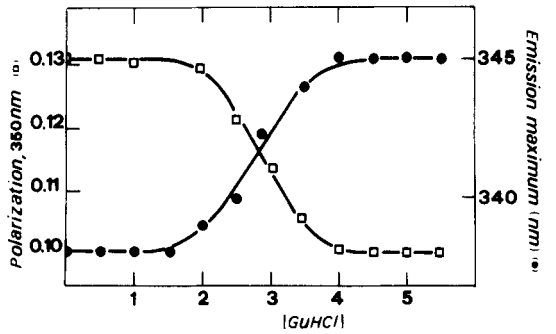

FIGURE 10: Effect of Gdn·HCl concentration on the tryptophanyl fluorescence polarization (□) and emission maximum (●) of rabbit lung INMT at pH 7.9 in 0.05 M phosphate and 0.15 M KCl. Excitation was at 295 nm. Protein samples were prepared as reported in Figure 4.

concentration, the tryptophanyl emission is not yet completely normalized. Full normalization of the INMT emission spectrum, i.e., emission peak at 350 nm or longer wavelength, is attained only by adding to the enzyme dissolved in 6.0 M Gdn·HCl an excess of reducing agent such as MSH or DTT.

(*b*) *Tryptophanyl Fluorescence Polarization.* The dependence of the tryptophanyl fluorescence polarization at 350 nm on Gdn·HCl concentration is shown in Figure 10. Between 0 and 2.0 M Gdn·HCl, the degree of polarization was unchanged, and it was found to be around 0.13. The increase of Gdn·HCl concentration from 2.0 to 4.0 M produced a decrease of the tryptophanyl fluorescence polarization from 0.13 to 0.10. No further change of polarization was observed at Gdn·HCl concentrations higher than 4.0 M. The polarization change observed upon increase of denaturant concentration to 4.0 M indicates that the internal flexibility of the protein matrix is enhanced; however, the value of the tryptophanyl fluorescence polarization in strongly denaturant solvent is still higher than that observed for a low molecular weight model compound in water or for fully denatured proteins (Alexander et al., 1978). A further decrease of tryptophanyl fluorescence polarization (0.06) was obtained only by adding reducing agents (MSH or DTT) to the enzyme dissolved in 6.0 M Gdn·HCl, i.e., the same experimental conditions which were used to obtain full normalization of the tryptophanyl emission maximum. Therefore, it is reasonable to admit that the guanidine-unfolded INMT still retains a certain amount of organized structure which can be eliminated by the effect of reducing agents.

(*c*) *Enzyme Activity.* The dependence of the catalytic activity of rabbit lung INMT on Gdn·HCl concentration has been determined by using tryptamine as substrate. The results are reported in Figure 9. Guanidine hydrochloride inhibits the enzyme activity at very low concentrations, 0.1 M Gdn·HCl being able to produce 50% inhibition. It is evident that the data reported in Figures 9 and 10 indicate that the loss of the catalytic activity occurs as a separate process preceding the exposure of the tryptophanyl side chains to the solvent. Moreover, the loss of enzyme activity seems to be related to some extent to the fluorescence change observed at low guanidine concentrations, even though the two transition curves are not completely superimposable (Figure 9). Probably this fluorescence change reflects some minor structural change which is able to destroy the catalytic site of the enzyme but does not affect the overall shape of the molecule as indicated by the fact that the tryptophanyl fluorescence polarization is constant in the same range of denaturant concentration. The difference observed in the midpoints of the two transition curves could be due to a specific effect of the denaturant molecules on the substrate binding.

Discussion

INMT has been purified for the first time to apparent homogeneity from rabbit lung, chosen as the biological source because of its high content of such enzyme. The kinetic studies performed with the purified enzyme confirmed the previous results obtained with partially purified INMT preparations (Mandel et al., 1971; Wyatt et al., 1973; Borchardt, 1974; Porta et al., 1977a).

The rabbit lung INMT prepared according to our procedure consists of a single homogeneous polypeptide chain, the molecular weight of which is 31 500 ± 1000 as determined by several techniques. The ellipticity of the peptide chromophore is weak, thus indicating that there are only few groups in organized structure, essentially in the α-helical conformation. However, the protein is not characterized by a flexible structure like a randomly coiled polypeptide since its frictional ratio (1.05) is strongly indicative of a compact globular structure.

The stability of INMT has been evaluated by observing the effects produced by guanidine and acid on several structural parameters. The action of guanidine depends on its ability to disrupt hydrophobic interactions and to increase the solubility of the peptide group (Nozaki & Tanford, 1963, 1970). The effect of guanidine on the fluorescence intensity appears to be rather complex because of the concurrence of two overlapping phenomena. The fluorescence decrease observed at low Gdn·HCl concentrations (0–2 M) is not produced by a large conformational change since other molecular parameters which reflect the overall protein structure are not modified by the same denaturant concentration. On the other hand, the fluorescence decrease appears to be concomitant with the loss of catalytic activity. Therefore, it may be concluded that the active site of the enzyme is destroyed by a subtle structural rearrangement of the protein molecule without a large change in its size and shape. This conclusion is also in agreement with the observations made by several authors that the catalytic site of many enzymes is less stable and close-packed than the remainder of the protein matrix (Cockle et al., 1978a,b).

A major structural transition with abrupt unfolding takes place as the Gdn·HCl concentration increases from 2.0 to 4.0 M. The transition is sigmoidal, but its broadness is indicative of low cooperativity, or it might represent the product of several overlapping independent transitions. However, the final state reached in concentrated guanidine solutions (6.0 M) shows some spectral features which are typical of polypeptides containing indole residues not completely exposed to the solvent or freely rotating. Full normalization of the spectral parameters is obtained only in the presence of reducing agents in denaturant solvent. Therefore, it is likely that the molecular structure of INMT in 6.0 M Gdn·HCl is that corresponding to a disulfide cross-linked randomly coiled polypeptide. The presence of very small regions of organized structure which survive the guanidine-induced structural transition is not excluded. In fact, the indole residues are sufficiently constrained so that their emission maximum as well as their degree of fluorescence polarization is significantly different from that which would prevail in the absence of constraints. It has been shown that constraints affecting the rotational freedom of a given residue occur when the residue is within closed rings which are composed of no more than eight to ten residues (Tanford, 1968). Therefore, if disulfide bonds give rise to closed rings including the indole residues of the enzyme molecule, the rings have to be small enough to reduce the

rotational freedom of the chromophore.

Acid is known to be a less effective protein denaturant, even though many proteins appear to be so thoroughly altered by acid that the final product is indistinguishable from a random coil (Tanford, 1968). INMT does not appear to reach a random-coiled state in a strongly acidic solution. In fact, the CD spectrum of the enzyme obtained at pH 2.0 largely differs from that calculated for a true randomly coiled polypeptide (Chen et al., 1974). The transition curve obtained following the CD activity at 222 nm clearly indicates the occurrence of two structural transitions. The first transition, which takes place between pH 6.5 and pH 4.5, produces a decrease of the α-helical content with no change in the degree of exposure of the tryptophanyl residues. The second one, occurring between pH 4.0 and pH 2.0, makes the protein able to bind ANS and slightly decreases the amount of organized structure. These findings might be explained by assuming that the globular structure of the native enzyme results from the close package of organized regions, which are joined by structureless segments. Conformational changes affecting the interactions among these regions would not shift the emission maximum if tryptophans are located within them. Moreover, a reorganization of the structured regions could be responsible for the ANS binding observed at low pH values as well as of the loss of catalytic activity observed at low guanidine concentrations.

As far as the enzyme activity is concerned, it has to be pointed out that the pH dependence of catalytic activity observed in our previous studies (Porta et al., 1979c) is not dependent on conformational changes. In fact, acidification from pH 8.0 to pH 6.0 produces 70% enzyme inactivation whereas, in the same pH range, neither the fluorescence intensity and maximum nor the far-ultraviolet CD spectrum was modified. Thus, it is likely that the enzyme activity is related to some group(s) that, if protonated, seriously affect(s) the enzyme function.

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