mequiv of Rh) of μ-dichlorotetraethylenedirhodium(I) and 288 mg (0.412 mequiv) of polymer **7** was placed in a Schlenk tube and the vessel was freed from oxygen by alternately evacuating and charging with dry and deoxygenated nitrogen. Benzene (4 mL) was added, the mixture was stirred for 10 min, and 8 mL of ethanol was added. The mixture was stirred at ambient temperature for 24 h under a stream of nitrogen to afford a light brown polymer-attached Rh catalyst **8**. Following the addition of 665 mg (5.15 mmol) of α-acetamidoacrylic acid under nitrogen, the reaction mixture was charged with hydrogen by alternately evacuating and then filling with hydrogen. The uptake of hydrogen was measured with a graduated cylinder connected to the reaction vessel, and the hydrogenation was run under 1 atm at 25 °C. The uptake of hydrogen ceased in 2 h. The polymer catalyst was filtered, the solvents were evaporated from the filtrate, and the residue was dried under reduced pressure. Conversion was measured by ^{1}H NMR and optical yield was determined by use of a polarimeter with reference to the value for the authentic sample, conversion 100%, optical yield 75.0%.

In the recyclization reaction, polymer filtration and all other procedures for the reaction were performed under nitrogen or hydrogen, and exposure to oxygen was strictly precluded.

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Measurement of Hydrogen Exchange at the Tryptophan Residues of a Protein by Stopped-Flow and Ultraviolet Spectroscopy

Mamoru Nakanishi,[1a] Hitomi Nakamura,[1a] Akiko Y. Hirakawa,[1a] Masamichi Tsuboi,*[1a] Toshihiko Nagamura,[1b] and Yutaka Saijo[1b]

Contribution from the Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan, and Union Giken Co., Ltd., Osaka, Japan. Received March 23, 1977

Abstract: A time-dependent change in the ultraviolet absorbance at 290 nm of the indole ring of tryptophan has been observed, using a stopped-flow spectrophotometer, when tryptophan was rapidly transferred from water into deuterium oxide–water solution. From this experiment, the rate constant of the hydrogen–deuterium exchange reaction of the tryptophan NH group has been determined at various pH values and at several temperatures. Stopped-flow ultraviolet spectroscopy has also been used for an examination of hydrogen exchange kinetics of the tryptophan residues of hen egg-white lysozyme. Three of the six tryptophan residues of this protein molecule were deuterated in 50 min at pH 5.5 and 22 °C. These three residues are probably Trp-62, Trp-63, and Trp-123. When *N*-acetylglucosamine was present the deuteration rates were markedly lower.

Since we have accumulated knowledge on the hydrogen exchange reactions of the main-chain NH groups of several proteins,[2-5] including hen egg-white lysozyme,[2,3] it is now desirable to extend such knowledge to the side chains. It has been shown that hydrogen–deuterium exchange reactions at the tryptophan residues in a protein can be examined by proton magnetic resonance measurement[6] and also by Raman spectroscopic measurement.[7] In this paper, we demonstrate that this can be done by stopped-flow ultraviolet spectroscopy, similarly to Cross's method for a few nucleosides.[8,9] This method has a few advantages in comparison with the other two. First, it can be applied to a fast exchange reaction which takes place in a few milliseconds. It is applicable to a high molecular weight protein, whereas the proton magnetic resonance method would not be. An ultraviolet absorption measurement, in addition, allows us to reach a sufficient signal-to-noise ratio with a relatively small amount of sample. We have applied this method to hen egg-white lysozyme, and the hydrogen-exchange kinetics of relatively labile tryptophan residues were examined both in the native, free state and in the inhibitor–enzyme complex.

Experimental Section

L-Tryptophan was purchased from Wako Pure Chemical Industries, Ltd., and six times recrystallized hen egg-white lysozyme was obtained from Seikagaku Kogyo Co., Ltd. The sample of human lysozyme used was kindly provided by Professor K. Hamaguchi, Osaka University. Deuterium oxide (99.75 atom %) was purchased from Merck.

The hydrogen and/or deuterium ion concentrations of the solution (H_2O, D_2O, or $H_2O + D_2O$) were measured with a Toa Dempa pH
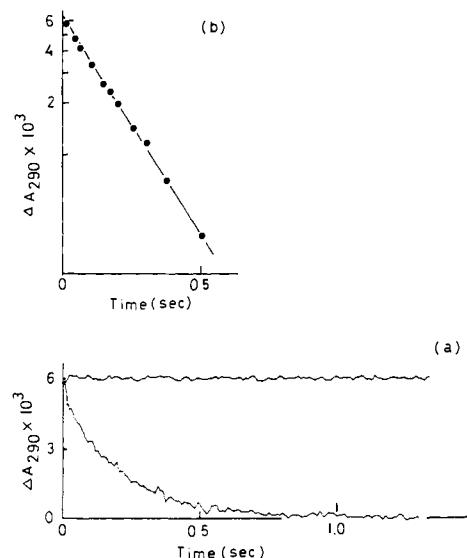
Figure 1. (a) The time dependence of the decrease in absorbance at 290 nm observed when L-tryptophan dissolved in H_2O (0.05 M phosphate buffer, pH 7.0, 22 °C) is mixed with D_2O (1:1 in volume; final concentration of L-tryptophan is 0.26 mM). (b) Semilogarithmic plot of the same data. (In a, a curve, nearly straight, running parallel to the time axis, which was recorded in our control experiment, is also shown. This is an experiment in which the absorbance at 290 nm was observed when the same tryptophan solution as above was mixed with H_2O, instead of D_2O. Here, the absorbance was found to remain unchanged.)

Figure 2. Solvent perturbation difference spectra observed when the spectrum of L-tryptophan (0.26 mM) in H_2O is compared with that of L-tryptophan in $H_2O + D_2O$ (1:1 in volume; pH 5.5, 22 °C): (a) the difference spectrum obtained from an equilibrium measurement; (b) the points are the total absorbance change obtained for the first-order process observed on dilution with D_2O ($H_2O:D_2O = 50:50$).

meter and a Hitachi-Horiba 7SS pH meter. In this paper, we use the notation "pH" even for the deuterium ion concentration of a deuterium oxide solution, and pH metric readings are always given without any correction. For adjusting and keeping each pH value, 0.05 M phosphate buffer was used (final concentration after mixing, 0.025 M).

Ultraviolet absorption and difference spectra were observed by the use of a Hitachi EPS-3T recording spectrophotometer and also by the use of a Union Giken high-sensitivity spectrophotometer SM-401. Fast exchange reactions of 100 ms to 50 s in the time scale were traced by the use of a Union Giken stopped-flow spectrophotometer RA-401. This is equipped with a rapid-mixing device of a dead time of 500 μs (determined by the method of Hiromi et al.[10]), with a cell of optical path length 10 mm, and with an ultraviolet spectrophotometer of focal length 25 cm, sensitivity 0.0004 OD unit, and response time of 1 μs. This was connected with a Union Giken data-processor RA-450, a monitor scope, and an XY recorder. Slower exchange reactions were traced by another stopped-flow system with a dead time of 20 ms and a Union Giken SM-401 or a Hitachi 124 spectrophotometer.

Results

Hydrogen–Deuterium Exchange in Free Tryptophan. When L-tryptophan in H_2O is rapidly mixed with D_2O (at pH 7.0, 22 °C), a time-dependent decrease in absorbance at 290 nm is observed as shown in Figure 1a. A replot of such data, as illustrated in Figure 1b, shows that the absorbance decrease takes place as a first-order process. By extrapolating the straight line in Figure 1b to zero time, we are able to determine the total absorbance change associated with this first-order process. The total absorbance changes measured in this way at several different wavelengths generate the kinetic difference spectrum shown in Figure 2. In this figure, the equilibrium solvent perturbation difference spectrum, which gives the total effect of D_2O on the spectrum of L-tryptophan, is also shown. The difference between the kinetic difference spectrum and equilibrium solvent perturbation difference spectrum is attributed to a solvent perturbation which takes place during the dead time of the kinetic experiment. The equilibrium solvent perturbation difference spectrum has three peaks at about 291, 283, and 274 nm. Of these three, only the 291-nm peak is involved in the time-dependent process, while the 283-nm peak
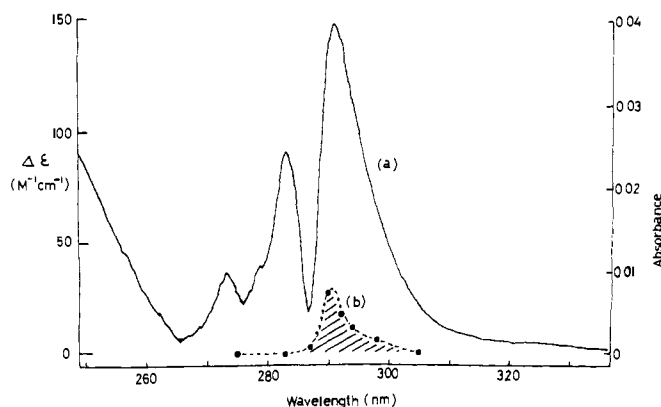
does not seem to be involved. This is to be explained by taking the isotope effects on the vibronic levels into consideration. The magnitude of the perturbation difference spectrum observed here is in an agreement with what was observed by Herskovits and Sorensen.[11]

The first-order process shown in Figure 1 is now attributed to the hydrogen–deuterium exchange reaction of the indole NH group of tryptophan. The rate constant is found to be 6.0 s^{-1} (290 min^{-1}) at pH 7.0 and 22 °C. This is considered to be the rate constant k_e of a tryptophan residue which is completely exposed to the solvent, and such a value is useful as a reference in a study of the tryptophan residues involved in a protein molecule.

Similar observations were made at various pH values, and the observed rate constants, k_e, are given in Figure 3. The rate constants k_e were also determined at different temperatures (at pH 5.5). An Arrhenius plot is shown in Figure 4. The observed points fall on a straight line, and from the slope of this line the activation energy of the hydrogen–deuterium exchange reaction of tryptophan is obtained as $\Delta H^{\pm} = 15.9$ kcal/mol.

The values of the hydrogen-exchange rate constants, k_e, for the free tryptophan residue were estimated by Waelder et al.[12] to be 0.15–0.25 s^{-1} at pH 4.7 and 27 °C, on the basis of their Fourier transform saturation recovery experiment (NMR). This is nearly in agreement with our present experimental results. Thus, our value $k_e = 0.087 s^{-1}$ (at pH 4.7 and 22 °C) is converted into $k_e = 0.14 s^{-1}$ (at pH 4.7 and 27 °C) by assuming that the activation energy $\Delta H^{\pm}$ is equal at pH 4.7 to that at pH 5.5. In our previous work,[7] on the other hand, greater values of k_e were given in the pH 3–7 range. These values are based upon the width measurement of the NH proton magnetic resonance signal in an H_2O solution. In light of the results of our present more direct kinetic measurements, however, we should reconsider the interpretation of the width of the magnetic resonance signal. The broadening of the NH signal observed in the pH 3–7 range must now be considered to be caused partly by some other factors than the hydrogen-exchange lifetime.

Hydrogen–Deuterium Exchange of the Tryptophan Residues in Hen Egg-White Lysozyme. Hen egg-white lysozyme in H_2O (initial concentration, 0.13%; final concentration, 0.065%) was mixed with the same volume of D_2O at pH 5.5 and 22 °C, and the time-dependent decrease in absorbance at 293 nm was observed. The result is shown by solid circles in Figure 5. This is indicative of the time course of the deuteration reaction of the tryptophan residues in the lysozyme molecule. In this plot, the ordinate scale (the number of tryptophan residues re-
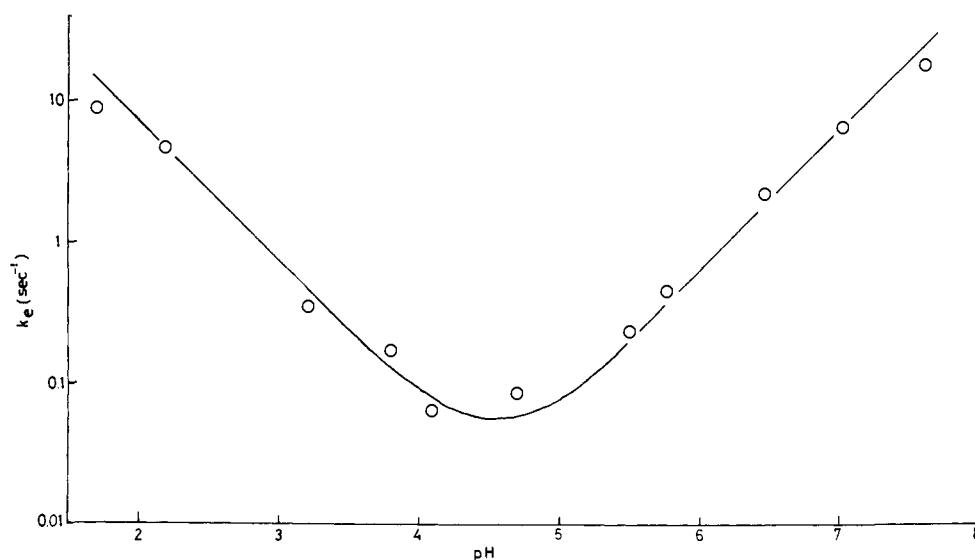
Figure 3. The rate constant k_e of the hydrogen–deuterium exchange reaction of tryptophan plotted on a logarithmic scale against the pH of the solution at 22 °C.
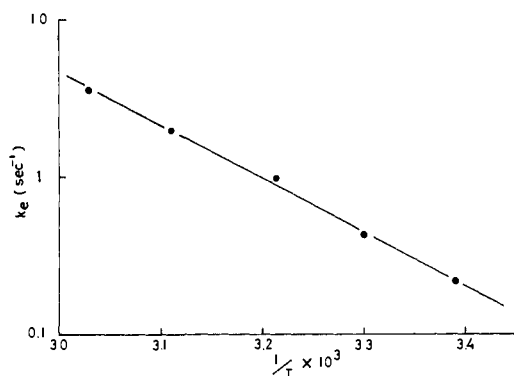

Figure 4. The rate constant k_e of the hydrogen–deuterium exchange reaction of tryptophan plotted on a logarithmic scale against reciprocal absolute temperature at pH 5.5 (Arrhenius plot).
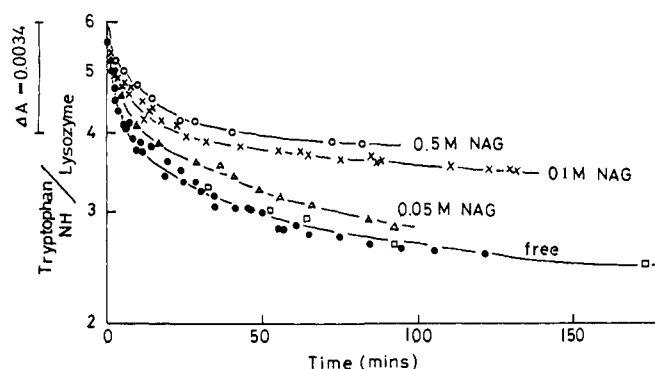

Figure 5. Semilogarithmic plots of the kinetic data for the hydrogen–deuterium exchange reactions at the indole NH groups of the tryptophan residues in hen egg-white lysozyme, pH 5.5, 22 °C. (●) Observed by the use of stopped-flow equipment with an ultraviolet spectrophotometer; 1:1 ratio drive syringes were used to give a final concentration of 0.045 mM of substrate-free lysozyme. (△, ✕, and ○) The same, except that *N*-acetylglucosamine (0.05, 0.1, and 0.5 M, respectively) was added to both of the mixing solutions. (□) Observed for 6.9 mM substrate-free lysozyme by the use of a Raman spectrophotometer. The intensity ratio of the 1386-cm^{-1} line (which appears only for deuterated tryptophan) vs. the 760-cm^{-1} line (which appears for both of the undeuterated and deuterated tryptophan residues) was examined as a function of time. The method was detailed by Takesada et al.[7] The continuous curves are the theoretical ones, calculated from the equation $H(t) = \Sigma_{j=1}^{6} \exp(-k_j t)$, where $H(t)$ is the number of the tryptophan NH groups remaining undeuterated at time t per molecule and k_j is the rate constant of the jth tryptophan residue. The k_j values assigned to Trp-28, -62, -63, -108, -111, and -123 are as follows: free lysozyme—0.0035, 0.65, 0.035, 0.0035, 0.0035, and 0.22 min^{-1}; with 0.05 M NAG—0.0, 0.20, 0.015, 0.0, 0.0, and 0.22 min^{-1}; with 0.1 M NAG—0.0, 0.10, 0.005, 0.0, 0.0, and 0.22 min^{-1}; with 0.5 M NAG—0.0, 0.03, 0.002, 0.0, 0.0, and 0.22 min^{-1}. Each of these sets is considered to be a possible set, but not the unique one.

maining undeuterated) was determined on the assumption that the absorbance difference, ΔA, at 293 nm between the completely undeuterated and completely deuterated lysozyme corresponds to six tryptophan residues, and that ΔA in any partially deuterated stage is proportional to the number of undeuterated tryptophan residues. The completely deuterated lysozyme was obtained by heating the D_2O + H_2O solution at 70 °C for 10 min and then cooling. The same deuteration process can also be followed by the use of Raman spectroscopy. As detailed by Takesada et al.[7] for the case of bovine α-lactalbumin, the time-dependent increase of the Raman intensity of the Raman line at 1386 cm^{-1} of lysozyme has been examined with the 760-cm^{-1} line as an internal standard. The result is also given in Figure 5 by open squares.

Of the six tryptophan residues of lysozyme (at positions 28, 62, 63, 108, 111, and 123), three at 28, 108, and 111 are known to be involved in intramolecular hydrogen bonds through the indole NH groups.[13,14] On the basis of the results of the proton magnetic resonance study of Glickson et al.[6] and of Campbell et al.,[15] it is probable that the rate constants of the hydrogen–deuterium exchange reactions of these three tryptophan residues are all smaller than 10^{-2} min^{-1} at pH 5.5 and 22 °C. Thus, the main part of the kinetics plot shown in Figure 5 is to be ascribed to the remaining three tryptophan residues, namely Trp-62, Trp-63, and Trp-123.

The Effect of Inhibitor Binding. An identical experiment to that just described, except that *N*-acetylglucosamine (NAG)

was added to the protein solution, has been made. The kinetics plots for such a case are also shown in Figure 5. With 0.1 M *N*-acetylglucosamine, for example, 80% of lysozyme molecules should have this inhibitor bound; the binding equilibrium constant at 22 °C is known[16,17] to be about 40 M^{-1}. As is seen in the figure, such a binding of *N*-acetylglucosamine causes a marked, general lowering of the hydrogen–deuterium exchange rates.

Human Lysozyme. Human lysozyme is known to have a similar chemical structure to that of hen egg-white lysozyme, but Trp-62 and Trp-123 in the latter are both replaced by ty-

rosine in the former.[18] Phe-34 in hen egg-white lysozyme is replaced by tryptophan in human lysozyme. A hydrogen-deuterium exchange reaction was examined of this protein at pH 5.5 and 22 °C. It has been found that all of the rate constants for five tryptophan residues are lower than 0.04 min^{-1}.

Discussion

The stopped-flow ultraviolet-absorption method has opened up a way of examining relatively fast hydrogen exchange reactions of the tryptophan residues in a protein molecule. As has already been mentioned, the deuteration processes observed for lysozyme in the 0–50-min period at pH 5.5 and 22 °C are assigned to Trp-62, Trp-63, and Trp-123. Of these three, Trp-63 is suggested to be rather slowly deuterated with a rate constant of $k < 0.04$ min^{-1} from our experiment on human lysozyme. Therefore, the remaining two, Trp-62 and Trp-123, may be considered to be the fastest. This view is in an agreement with what is expected from a crystallographic study of Phillips and coworkers.[13,14] Glickson et al.[6] estimated, in collaboration with Phillips, the surface area of the indole nitrogen accessible to water in the protein relative to that in small peptides. These are 0.55, 0.45, and 0.35, respectively, for Trp-62, Trp-123, and Trp-63 in the lysozyme molecule. It should be pointed out here that even the fastest tryptophan in lysozyme has a rate constant value (about 0.7 min^{-1} or less, at pH 5.5 and 22 °C) lower than that of completely free tryptophan by at least one order of magnitude.

Let us next discuss the effect of inhibitor binding on the hydrogen exchange rates of the tryptophan residues of lysozyme. An x-ray crystallographic study[13,14] indicates that the indole NH groups of Trp-62 and Trp-63 form hydrogen bonds with oxygen atoms of N-acetylglucosamine, while Trp-123 seems to be indifferent to the inhibitor binding. We may therefore expect that one of the fastest two rate constants would be greatly lowered whereas the other would remain unchanged, when N-acetylglucosamine is bound by this enzyme molecule. The question now is whether this expectation fits with our experimental results.

Let us consider an extreme case (case i) where a tryptophan residue cannot be deuterated at all when N-acetylglucosamine is bound to lysozyme. In this case the hydrogen–deuterium exchange rate constant k (with NAG) should be given by:

$$k(\text{NAG}) = \frac{[\text{E}]}{[\text{E} \cdot \text{NAG}] + [\text{E}]} k(\text{free}) \qquad (1)$$

Hence:

$$\frac{k(\text{free})}{k(\text{NAG})} = K_a[\text{NAG}] + 1 \qquad (2)$$

where K_a is the association constant which is assumed to be equal to 40 M^{-1} at pH 5.5 and 20 °C.[16,17] Let us consider another extreme case (case ii), where the rate constant (k) of a tryptophan residue does not change at all even when N-acetylglucosamine is bound to the lysozyme molecule. In this case, of course:

$$\frac{k(\text{free})}{k(\text{NAG})} = 1 \qquad (3)$$

In Figure 6, theoretical curves of $k(\text{free})/k(\text{NAG})$ vs. [NAG] for cases i and ii are shown.

Instead of analyzing in detail each of the kinetics plots in Figure 5, we have determined the time ($\tau_{2/6}$) required for deuterating two tryptophan residues out of six in the lysozyme molecule. This is easily done by drawing a straight line in Figure 5 through the abscissa 4 running parallel to the time
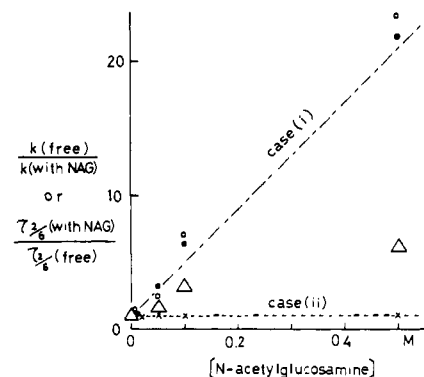

Figure 6. The ratio of the rate constant k_j(free) for lysozyme vs. k_j(NAG) for lysozyme + N-acetylglucosamine plotted against the total concentration of N-acetylglucosamine (which is practically equal to the concentration of free N-acetylglucosamine): (- · -) theoretical curve for case i (see text). (· · ·) theoretical curve for case ii. (△) observed $\tau_{2/6}$(NAG)/$\tau_{2/6}$(free); (●, ○ and ✕) k(free)/k(NAG), calculated by the use of the rate constant values assigned to Trp-62, Trp-63, and Trp-123, respectively, on the basis of the experimental results given in Figure 5. These rate constant values from one of the possible sets, but are not necessarily unique.

axis, and then finding the point where it crosses the kinetics curve. Now:

$$\frac{\tau_{2/6}(\text{NAG})}{\tau_{2/6}(\text{free})} = \left\langle \frac{k(\text{free})}{k(\text{NAG})} \right\rangle \qquad (4)$$

where the angular brackets indicate an average value of $k(\text{free})/k(\text{NAG})$, to which most of the contribution is made by Trp-62 and Trp-123, but a slight contribution may also be made by Trp-63 and other three tryptophan residues. The observed values of $\tau_{2/6}(\text{NAG})/\tau_{2/6}(\text{free})$ are plotted as triangles in Figure 6. As may be seen here, the observed points fall in between the area of the theoretical curves for cases i and ii. This fact is not contradictory with the view that $k(\text{free})/k(\text{NAG})$ values for Trp-62 are on the case i curve and $k(\text{free})/k(\text{NAG})$ values for Trp-123 are on the case ii curve. In fact, it is found to be possible to assign a set of rate constant values to Trp-62 and Trp-123, as well as to Trp-63 and other tryptophan residues in a self-consistent manner, so that the kinetics plots in Figure 5 are all well reproduced. At the same time in the $k(\text{free})/k(\text{NAG})$ vs. [NAG] plot the points for Trp-62 and Trp-63 fall on the case i theoretical curve, whereas those for Trp-123 fall on the case ii theoretical curve (see Figure 6).

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Role of 1,2-Dehydroreticulinium Ion in the Biosynthetic Conversion of Reticuline to Thebaine

Paul R. Borkowski, Jerold S. Horn, and Henry Rapoport*

Contribution from the Department of Chemistry and Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. Received June 27, 1977

Abstract: The role previously assigned to 1,2-dehydroreticulinium ion as a precursor to the morphinan alkaloids in *Papaver somniferum* was based on feeding experiments with a synthetic compound of uncertain identity. We have now prepared authentic 1,2-dehydroreticulinium chloride and shown its efficient incorporation into the morphinan alkaloids, supporting the previous hypothesis. Moreover, using a double-label technique and steady-state $^{14}CO_2$ biosynthesis, we have determined that 1,2-dehydroreticulinium ion is a natural product whose native pool size is about one-fifth that of reticuline. These data clearly establish 1,2-dehydroreticulinium ion as an intermediate in morphinan alkaloid biosynthesis.

Reticuline (**1**) has been firmly established[1,2] as the biosynthetic precursor, via salutaridine (**2**) and salutaridinol (**3**), of thebaine (**4**) in *Papaver somniferum*. Although it is the (−)-enantiomer of reticuline which corresponds in absolute stereochemistry to the configuration found at that center in the morphinan alkaloids,[3] both enantiomers, when fed separately, were incorporated into thebaine essentially to the same extent. Feedings with reticuline labeled with ^3H at C-1 and ^{14}C at other positions showed that incorporation into thebaine was accompanied by no loss of ^{14}C but with considerable loss of ^3H.[1b,c]

These unexpected results were accommodated by proposing a reversible oxidation–reduction side path to 1,2-dehydroreticulinium ion (**5**), which would allow both for the loss of ^3H and inversion of configuration at C-1. Support for this proposal was found when synthetic material characterized as 1,2-dehydroreticulinium chloride was efficiently incorporated into morphine,[1c] and dehydroreticulinium ion was assigned a role as a precursor of thebaine.

High incorporation is necessary and strong evidence for a precursor–product relationship; however, by itself it is insufficient. An additional requirement is the natural occurrence of the candidate precursor, a question which we set about to answer for 1,2-dehydroreticulinium chloride (**5**). The only characterization for **5** previously reported[1c] was its mp (190–200 °C dec) and ultraviolet absorption. Since the latter (λ_{max} 250, 323 nm) did not correspond to that of similar 1-benzyl-3,4-dihydroisoquinolinium salts in our experience nor to published spectra,[4] it also was necessary to prepare fully authenticated 1,2-dehydroreticulinium chloride and reexamine its role as a precursor.

Discussion

Synthesis of 1,2-Dehydroreticulinium Chloride (5) and Its ^3H and ^{14}C Isotope Isomers.

1,2-Dehydroreticulinium ion is a common intermediate in the many syntheses of reticuline[5] and our synthesis proceeded by standard methods from vanillin to 3-benzyloxy-4-methoxyphenylacetic acid (**6**). For the other half of the molecule, we chose 4-benzyloxy-3-methoxyphenylacetonitrile (**7**) as the intermediate, anticipating the intro-

duction via the cyano group of ^{14}C and ^3H at C-3 of the final compound.

The conversion of nitrile **7** to amine **8** has been erratic in the past using the more conventional (PtO_2/H_2 or $LiAlH_4$) methods. We found that this reduction can be reliably performed and in good yield using sodium borohydride and cobalt chloride in methanol.[6] The amide **9** then formed nearly quantitatively when acid **6** and amine **8** were refluxed in xylene with removal of water. Cyclization of the amide in refluxing toluene with $POCl_3$ gave the iminium chloride **10** in 95% yield. When **10** as the free base was treated with methyl iodide in methanol, a 92% yield of the methiodide **11** resulted. To prepare the corresponding chloride, **11** was treated with excess freshly prepared AgCl in aqueous methanol, giving quantitative conversion to methochloride **12**. Finally, debenzylation of **12** occurred quantitatively in refluxing ethanolic HCl to give pure 1,2-dehydroreticulinium chloride (**5**) (see Scheme I).

In our hands, 1,2-dehydroreticulinium chloride (**5**) is a stable salt melting at 180–185 °C dec. The infrared spectrum shows clearly a band at 1630 cm^{-1} characteristic of the conjugate iminium salt. The ultraviolet spectrum (λ_{max} 370, 309, 250 nm) of **5** is in accord with the earlier report on the ultraviolet spectra of dihydroisoquinolines of this type,[4] but contrasts with the previous[1c] absorption (λ_{max} 323, 250 nm) assigned to structure **5**. The NMR spectrum accounts for all the hydrogens of **5** and confirms its iminium salt character (as shown in the infrared) by the appearance of the C-9 methylene hydrogens as a two-hydrogen singlet at δ 4.40. The NMR spectrum can be regenerated sans the δ 4.40 signal if the sample is treated with NaOD in D_2O to pH 13 and immediately quenched by acidification to pH 2 with DCl in D_2O. Finally, 1,2-dehydroreticulinium chloride (**5**) was reduced with sodium borohydride to give an excellent yield of D,L-reticuline which was further characterized by conversion to its perchlorate and picrate salts, in full agreement with the reported data.[5]

To prepare [3-^{14}C]reticuline and [3-^{14}C]-1,2-dehydroreticulinium chloride (**5**) Na^{14}CN was used in the reaction with 4-benzyloxy-3-methoxybenzyl bromide, and the resulting radioactive acetonitrile **7** was carried through the synthesis as