In the aforementioned experiment, as well as in all other studies of this sort,[1] the solvent was selected solely on the basis of such criteria as maximal enzymatic conversion rates and reactant solubilities; the possibility of affecting the enzyme enantioselectivity by the solvent was not considered. Encouraged by our recent findings,[8] we proceeded to examine the dependence of v_S, v_R, and their ratio (the enantioselectivity factor) on the reaction medium for one of the enzymes tested above, subtilisin. As seen in Table I, subtilisin's enantioselectivity factor is a strong function of the solvent and varies from near unity for toluene, octane, or cyclohexane to 7.7 for 3-methyl-3-pentanol. An even greater effect was observed for another chiral amine, 1-(1-naphthyl)ethylamine, for which the v_S/v_R ratio under the same conditions was 1.6 in octane and 22 in 3-methyl-3-pentanol.

The marked enantioselectivity expressed by subtilisin in 3-methyl-3-pentanol is of a magnitude that should be sufficient for kinetic resolutions of racemates.[9] Therefore, we employed subtilisin (a readily commercially available enzyme) in this solvent for preparative resolution of numerous racemic amines. Inspection of Table II reveals that the enzyme invariably exhibits a profound *S* selectivity and consequently optically active amides and amines have been prepared on a millimole scale with good, in most cases, enantiomeric excess.

Our findings demonstrate that screening is not the only route to enantioselective enzymes, for a nonselective enzyme in one solvent may become a useful selective catalyst in another. The scope and mechanism of this phenomenon are under investigation.

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Protochlorophyllide Reductase. 1. Determination of the Regiochemistry and the Stereochemistry of the Reduction of Protochlorophyllide to Chlorophyllide

Tadhg P. Begley* and Helen Young

Department of Chemistry, Cornell University
Ithaca, New York 14853
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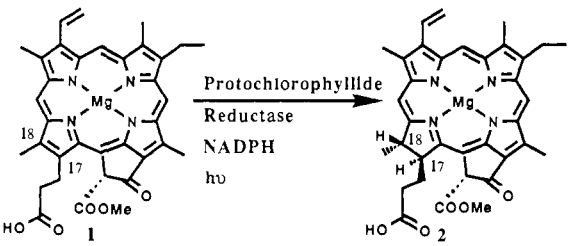
It has been estimated that over 1 billion tons of chlorophyll are synthesized in the biosphere each year.[1] Although the chlorophyll biosynthetic pathway has been the subject of intense investigation over the past 50 years, much remains to be discovered.[2,3] One promising area of investigation is the remarkable color change, from yellow to green, that occurs when dark-grown plants are exposed to light. This "greening reaction" involves the reduction of protochlorophyllide **1** to chlorophyllide **2** and is a key regulatory step both in the biosynthesis of chlorophyll and in the development of the chloroplast[4-6] (eq 1). This reaction is catalyzed by the



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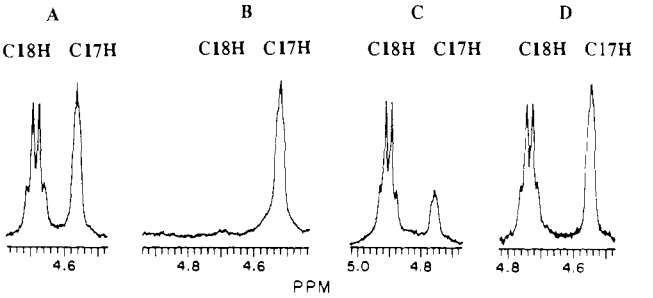

Figure 1. Partial NMR spectrum (400 MHz) showing the C17 and the C18 protons of pheophorbide: (a) control sample formed in protiobuffer, (b) sample formed in deuteriated buffer, (c) sample formed with [(4*S*-^{2}H)NADPH] as substrate, and (d) sample formed with [(4*R*-^{2}H)-NADPH] as substrate.

enzyme protochlorophyllide reductase and is of interest because it is one of only two known light-requiring enzymatic reactions.[7] The enzyme has been purified from oats,[8] barley,[9] wheat,[10] and squash.[11] It is an unstable membrane-bound protein of 37 000 D molecular weight.[8] The quantum yield for protochlorophyllide reduction in barley seedlings is 0.4[12] and the action spectrum for the reaction in corn seedlings follows the absorption spectrum of protochlorophyllide.[13] As the first step in our mechanistic investigation of this important reaction, we have determined the regiochemistry and the stereochemistry of the reduction catalyzed by the oats enzyme. On the basis of this information, we propose a model for the active site of the enzyme.

Enzymatic reactions were run by using a modification of the Griffiths procedure.[14] The reaction mixture consisted of 34 μM protochlorophyllide, 0.75-1.5 mM NADPH, and etioplast membranes (2-4 mg of protein/mL) suspended in 50 mL assay buffer. Protochlorophyllide was isolated from etiolated oat seedlings.[14] [(4*R*-^{2}H)NADPH] was prepared by the alcohol dehydrogenase-catalyzed reduction of $NADP^+$ by 2-propanol-d_8 (from *Thermoanaerobium brockii*).[15] [(4*S*-^{2}H)NADPH] was prepared by the glucose-6-phosphate dehydrogenase catalyzed reduction of $NADP^+$ by glucose-6-phosphate-1-*d*.[16] Etioplast membranes were isolated from 200 g of 7 day old etiolated oat seedlings.[14] These preparations typically contained 60% protochlorophyllide reductase and had a specific activity of 0.25 nmol/mg/flash (averaged over 23 flashes). After 23 flashes (1 flash/40 s) the reaction was quenched with acetone and extracted with ether. The chlorophyllide was then demetalated with hydrochloric acid (20 mM) and the resulting pheophorbide purified by HPLC. The purified product (70-120 μg) was analyzed by ^{1}H NMR.

The region of the ^{1}H NMR spectrum showing the C17 proton and the C18 proton resonances of pheophorbide is shown in Figure 1A.[17] The position of these signals varied between experiments, presumably due to variations in the pheophorbide concentration.[18,19] The relative integration (C18H:C17H) for the control sample was 1:0.8. The level of deuterium incorporation was

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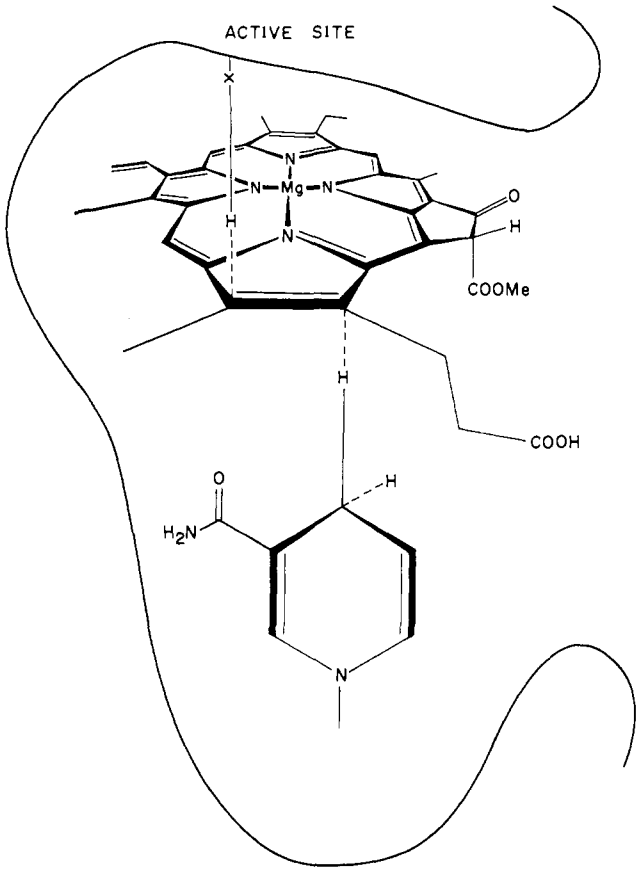

Figure 2. Model for the active site of protochlorophyllide reductase.

calculated relative to the control integration. When the enzymatic reaction was run in deuteriated assay buffer, the C18 proton resonance was almost completely depleted (Figure 1B). When the enzymatic reaction was run with [(4*S*-^{2}H)NADPH][20] as the cofactor, the C17 proton resonance was 70% depleted (relative integration 1:0.24, Figure 1C). Finally, when the reaction was run with [(4*R*-^{2}H)NADPH] as substrate, the NMR spectrum was indistinguishable from that of the Control[21] (Figure 1D). As the coupling between the C17 proton and the C18 proton is small (2 Hz[17]), deuteriation results in only minor changes in the splitting pattern. It has been previously demonstrated that the barley enzyme also catalyzes the transfer of the *pro-S* hydride from NADPH.[15] A possible rationale for the incorporation of only 70% deuterium at C17 is currently under investigation.

These studies suggest the model for the active site of protochlorophyllide reductase that is outlined in Figure 2. The fact that the enzyme catalyzes a trans reduction follows from the structure of chlorophyll. Regarding the origin of the hydrogen atoms, the NMR studies presented in this communication demonstrate that a hydride is delivered to the C17 position of protochlorophyllide from the *pro-S* face of NADPH and that the C18 position is protonated by water or an active site acid.[22]

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2D Chemical Exchange NMR Spectroscopy by Proton-Detected Heteronuclear Correlation

Gaetano T. Montelione and Gerhard Wagner*

Biophysics Research Division
Institute of Science and Technology
The University of Michigan
Ann Arbor, Michigan 48109-2099

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We have developed new heteronuclear 2D NMR experiments for characterizing slow dynamic equilibria in macromolecules. These methods allow determination of both equilibrium constants and exchange rates from integration of 2D NMR cross peaks and are therefore in this respect superior to existing homonuclear 2D exchange spectroscopy techniques which require integration of diagonal peaks for determination of equilibrium constants. Chemical exchange NMR spectroscopy provides information about equilibrium constants and rates of conformational isomerization for chemical systems which are in slow dynamic equilibrium.[1-3] To date, most chemical exchange studies have utilized one-dimensional NMR methods, which can be more sensitive than transient 2D experiments if steady-state irradiation is applied. However, 1D techniques generally have insufficient resolution for extensive dynamic studies of macromolecules. Several two-dimensional chemical exchange NMR experiments are available, including NOESY[4] (2D nuclear Overhauser effect and exchange spectroscopy), Camelspin or ROESY[5] (rotating frame NOE spectroscopy), and 2D zz-spectroscopy.[6] However, NOESY and ROESY are of limited utility in studies of macromolecules if the chemical exchange crosspeaks are close to the diagonal or overlapped with crosspeaks arising from dipolar coupling. Homonuclear zz-spectroscopy[6] provides exchange crosspeaks well-resolved from the diagonal, but these peaks are antiphase with respect to homonuclear couplings. Since the proton line widths of proteins are comparable to or larger than the homonuclear coupling constants, crosspeaks in homonuclear zz-spectroscopy suffer from conformation-dependent cancellation artifacts and cannot be used for quantitative measurements of equilibrium constants and exchange rates. The new experiments described here provide exchange crosspeaks which are not overlapped with a diagonal and, due to their in-phase multiplet structure, peak volumes which can be related directly to equilibrium constants and exchange rates. By utilizing heteronuclear correlation, these experiments have better resolution of direct peaks arising from multiple conformational states and of exchange crosspeaks due to magnetization transfer between these states than their homonuclear counterparts. When performed at natural ^{13}C or ^{15}N abundance, these experiments are exclusively sensitive to chemical exchange and exclude dipole–dipole magnetization transfer pathways.

The pulse sequence of Figure 1A provides information about chemical exchange of I_zS_z spin states[7] between different molecular

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