Stereoselective, Nonenzymatic, Intramolecular Transfer of Amino Acids†

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ABSTRACT: Biological systems synthesize proteins with an almost exclusive use of L-amino acids and virtually none of the D isomer. There has been no satisfactory explanation for the origin of this use of the L isomer. Research presented here shows that at pH 5, transfer of phenylalanine from the adenylate anhydride to ester occurs and is 95–97% efficient for the L isomer and only about 50% efficient for the D isomer. The origin of the use of the L isomer, given D-ribose nucleotides, may be based in part on this stereoselectivity.

Certainly one of the greater challenges in explaining the origin of our contemporary biological systems is that of elucidating the basis for the use of specific stereoisomers. With respect to the genetic system, all living forms use nucleic acids, with D-ribose, for the storage and transmission of information. The origin of this exclusive use of D-sugars has not been explained. Similarly, the expression of genetic information in contemporary biological systems involves the synthesis of protein consisting exclusively of L-amino acids. This exclusive use of L-amino acids is likewise unexplained.

Research on the origin of biological systems is generally based on the assumption that they evolved around possible chemical reactions and interactions. Consequently, to understand the origin of a biochemically entity, one can study the fundamental chemical steps involved. A good example of this approach is the work by Orgel and his co-workers (Orgel, 1987) aimed at understanding the origin of the processes of replication and/or transcription of nucleic acids. Put more broadly, they have studied information transfer from a preexisting nucleic acid strand by nonenzymatically synthesizing a complementary strand. While there are some restrictions on composition, it seems generally true that one can nonenzymatically make complementary copies of preexisting nucleic acid strands. These experiments unquestionably have shown that our contemporary processes of replication and transcription, including the retention of chirality, have evolved around the reality of stereospecific base pairing.

We would similarly like to come to some understanding of the basis for the origin of the process of protein synthesis. To do so, we must understand the basis for the origin of the various steps in the process. The essential steps are (1) activation of the amino acid with ATP, forming the adenylate anhydride, (2) passage of the amino acid to become the 2′-(3′)-ester of the 5′-AMP residue at the 3′ end of its cognate tRNA, and (3) formation of the peptide bond by reaction of the aminoacyl-tRNA with an adjacent peptidyl-tRNA ester. The chemistry of protein synthesis is thus the chemistry of aminoacyl (peptidyl)-AMP.

We have studied the activation step to a limited extent and find it does proceed nonenzymatically, though at a very slow rate, and there are differences in the activation rates of various amino acids (Mullins & Lacey, 1980a) and the activation rate is a direct function of the pK_a of the amino acid (Mullins & Lacey, 1980b). Furthermore, we have studied the transfer of amino acids from the adenylate anhydride either to become esterified to 5′-AMP itself (Lacey & White, 1972) or to the 2′-OH groups along the backbone of RNAs (Weber & Lacey, 1975) using imidazole as a transfer catalyst. The use of imidazole as a transfer catalyst may, in fact, be modeling the intermolecular enzymatic transfer of the contemporary process. The transfer of the amino acid from the anhydride to imidazole is quite rapid and efficient (Lacey & White, 1972).

We show in this paper, however, that a spontaneous, uncatalyzed intramolecular transfer of the amino acid from the adenylate anhydride to ester takes place. At pH 5, the transfer occurs with an almost quantitative yield using L-phenylalanine (L-Phe) but only half that for the D isomer. We believe the reality of this spontaneous transfer may be responsible for the origin of that step in the contemporary process of protein synthesis and the preference for L isomer may be one factor in the origin of the use of L-amino acids given D-ribose 5′-AMP.

Many years ago, Moldave et al. (1959) reported formation of ester in stored samples of adenylate anhydrides, but to our knowledge, no one has studied this reaction with any detail. Tyagi and Ponnamperuma (1990) did recently note the formation of esters in solutions of aminoacyl adenylate anhydrides but did not address the problem of chiral differences.

Experimental Procedures

Synthesis and Purification of Adenylate Anhydrides. All reagents were purchased from Sigma Chemical Co. The D- and L-Phe AMP anhydrides were synthesized as previously described (Lacey et al., 1984) using dicyclohexylcarbodiimide in aqueous pyridine at 0 °C. After precipitating the product with −20 °C acetone, it was filtered off and dissolved in water. This reaction appears to proceed (with Phe) with only a small amount of racemization. As synthesized, the product is, however, contaminated with unreacted 5′-AMP and the solvent pyridine. By application of the solution to a series of four Waters Sep-Pak (C_{18}) units, contaminating pyridine, AMP, phenylalanine, and acetone can be removed with a water wash, and the product is eluted with 100% methanol. Alternately, the anhydride can be purified by HPLC procedures described below with the experiments being done on the anhydride fraction after removal of salts with the Sep-Pak procedure.

Study of the Transfer Process. Experiments to study the transfer process were carried out at pHs 5.0, 5.3, and 5.7 with 0.05 M acetate buffer and with 0.05 M phosphate buffer at pH 6.1. Experiments were run with various adenylate concentrations from 1×10^{-5} to 1×10^{-3} M. The transfer reaction

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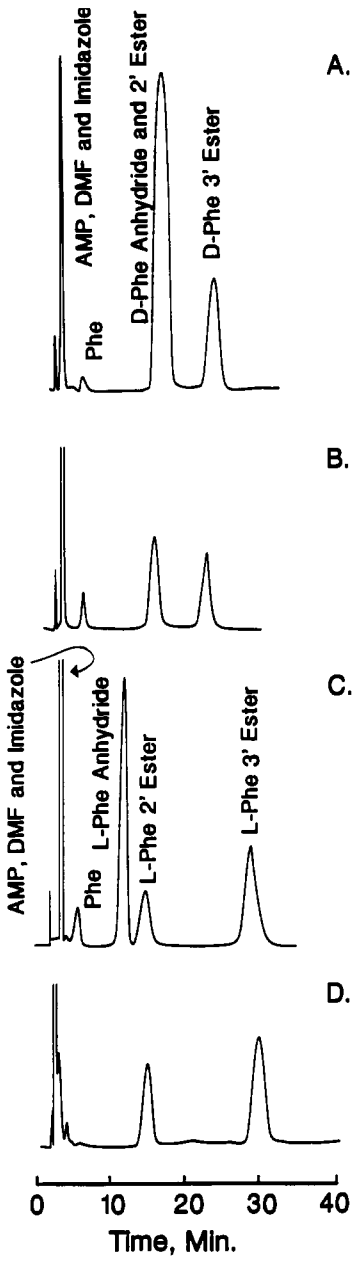

FIGURE 1: Reverse-phase (Waters phenyl Bondapak 10-μm particles) HPLC separation of D- and L-Phe AMP anhydrides and esters using 0.05 phosphoric acid (pH 2) at 1.0 mL/min. The ordinate is the absorbance at 259 nm. The column was 3.9 mm × 30 cm. (A) and (C) were mixtures of anhydrides and esters. (B) and (D) are respectivcly the D- and L-Phe AMP esters synthesized unambiguously as described in the text.

was followed by reverse-phase HPLC using a phenyl on silica column. The L-Phe adenylate anhydride and the 2′ and 3′ L-Phe AMP esters can be separated in this way (Figure 1C). Similarly, the D-Phe adenylate anhydride and the product 3′-ester can be separated (Figure 1A). The 2′ D-Phe AMP ester, however, elutes coincidentally with the D-Phe AMP anhydride. We could estimate the amount of 2′ D-Phe ester because both D- and L-amino acid esters of 5′-AMP reach an equilibrium distribution between the 2′- and 3′-positions. The D-Phe at pH 7 distributes 50% 2′ and 50% 3′ (Lacey et al., 1988). The present experiment involved lower pHs, so we determined the equilibrium distribution of the D-Phe ester at pH 6 and 5.5. Again we found approximately 50% 2′ and 50% 3′. Because we knew the 2′-ester of D-Phe is equal to the 3′-ester, we could carry out our study of the transfer process by running the HPLC of the D-Phe samples and doubling the amount of 3′-ester to estimate the total amount of D-Phe AMP ester. In the anhydride to ester transfer experiments, as the anhydride peaks decreased, the ester peaks increased. From integration of the peaks, the efficiency [Δ(ester)/Δ(anhydride)] was calculated. The various anhydride and ester peaks had been identified in earlier work by ^{1}H NMR.

We had earlier found that the hydrolysis of L-Phe adenylate anhydride is catalyzed by carbon dioxide (Lacey et al., 1984). The present studies were therefore carried out with buffer prepared with freshly boiled (30 min) water and under a nitrogen blanket. The pH was raised by bubbling nitrogen through a concentrated ammonia solution and then through a 1 N NaOH solution (to remove any CO_2) and finally into the sample. The pH was checked after the run to make sure it had remained constant. The amount of anhydride and ester present at 30 and 60 min was determined by the HPLC procedures mentioned above and in Figure 1. The pH studies were not carried below 5 because the rate of transfer process becomes progressively slower as the pH is lowered and only insignificant amounts of esters were formed in 30 min at pH 4.5.

Because both the starting anhydrides and the product esters are hydrolyzing in these experiments, we separately estimated the amount of hydrolysis taking place by following the amount of 5′-AMP appearing in the HPLC tracings as well as the amount of anhydride disappearing and ester appearing. This was only rigorously done at pH 5 where separate experiments were made and over a concentration range from 10^{-5} to 10^{-3} M. Rate constants were determined by plotting log % anhydride remaining vs time. The rate constant is 2.303 times the slope. There was no noticeable change in the rate constants for transfer and for hydrolysis with changes in concentration. Study of ester hydrolysis is described in the next section.

Synthesis, Purification, and Characterization of 2′,3′-Phenylalanyl-AMP Monoester. The product of the transfer, 2′,3′-phenylalanyl-AMP monoester, was synthesized by the method of Gottikh (1972) using *tert*-butoxycarbonyl-D- or L-phenylalanine. This intermediate was partially purified by the Sep-pak procedure above, the alcohol was evaporated off, and the *t*-Boc group was removed by reacting with 85% trifluoroacetic acid (TFA) for 10 min at 0 °C. The TFA was removed quickly under vacuum. The deblocked product was then purified by the Sep-Pak or HPLC procedures described above.

The rate of hydrolysis of this product was studied by incubation at pH 5 with 0.05 M acetate buffer. The hydrolysis rate at this pH was so slow for both the D- and L-esters that it was necessary to hydrolyze overnight. The hydrolysis rate constant was estimated from the log percent remaining vs time with data from HPLC analyses as described above.

Results and Discussion

The results of these transfer studies are presented in Figure 2 and plotted as the efficiency [Δ(ester)/Δ(anhydride)] at 30-min reaction time vs pH. The transfer of L-Phe at pH 5 proceeds with almost quantitative yield while the D-Phe transfer is only about 50% efficient. As the pH was increased, the efficiency of both isomers decreased because the hydrolysis rate of the starting anhydride and product ester increases exponentially with pH (Lacey et al., 1984).

The results in Figure 2 show the net result of triplicate experiments based on Δ(ester)/Δ(anhydride). It is possible that the results showing a higher efficiency of the L-isomer in the transfer are due to faster hydrolysis of the D-anhydride. To check this, separate triplicate experiments were carried out in which we followed the appearance of 5′-AMP, due to hy-

Table I: Rate Constants for the Total Disappearance of D- and L-Phe AMP Anhydride and for Ester Formation and Hydrolysis at pH 5 and 22 °C

compound	starting anhydride concn (M)	k_{total} (min^{-1})	$k_{hydrolysis}$ (min^{-1})	$k_{ester\ formation}$ (min^{-1})
L-Phe AMP anhydride	6.0×10^{-5}	15.35×10^{-4}	0.77×10^{-4}	14.58×10^{-4}
	6.0×10^{-5}	15.35×10^{-4}	0.77×10^{-4}	14.58×10^{-4}
	7.0×10^{-5}	15.73×10^{-4}	0.82×10^{-4}	14.91×10^{-4}
	1.0×10^{-3}	14.92×10^{-4} [a]		
		av 15.34×10^{-4} (SD = 0.33×10^{-4})	0.79×10^{-4} (SD = 0.029×10^{-4})	14.69×10^{-4} (SD = 0.19×10^{-4})
D-Phe AMP anhydride	6.0×10^{-5}	2.69×10^{-4}	1.53×10^{-4}	1.16×10^{-4}
	6.0×10^{-5}	2.69×10^{-4}	1.53×10^{-4}	1.16×10^{-4}
	1.3×10^{-4}	2.70×10^{-4}	1.62×10^{-4}	1.08×10^{-4}
	1.0×10^{-3}	2.66×10^{-4} [a]		
		av 2.69×10^{-4} (SD = 0.02×10^{-4})	1.56×10^{-4} (SD = 0.05×10^{-4})	1.13×10^{-4} (SD = 0.04×10^{-4})

[a] From the data in Figure 2. Hydrolysis data are not available for these experiments.

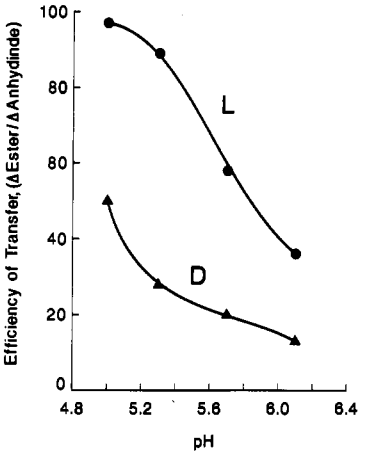

FIGURE 2: Efficiency of the anhydride to ester transfer [Δ(ester)/Δ(anhydride)] as a function of pH for L- and D-Phe at 30-min reaction time at room temperature (22 °C). Triplicate samples were run at each pH for each isomer, and the standard deviations in parentheses are as follows: L-isomer, pH 5.0 (1.2), pH 5.3 (1.9), pH 5.7 (0.6), pH 6.1 (2.0); D-isomer, pH 5.0 (4.4), pH 5.3 (3.5), pH 5.7 (2.1), pH 6.1 (1.4).

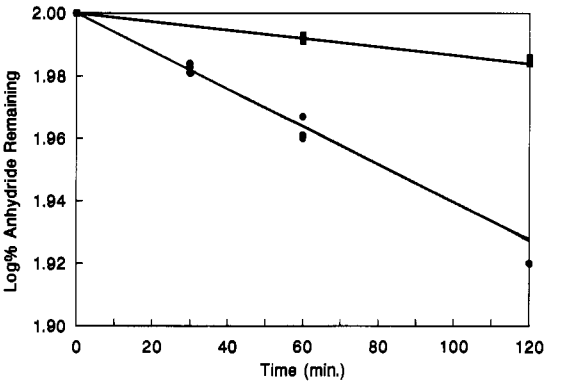

FIGURE 3: Plot of log percent original L-(●) and D-Phe AMP anhydride (■) remaining as a function of time in minutes (data in Table I). Experiments were at pH 5.0 and 22 °C. This disappearance is for ester formation plus hydrolysis. Hydrolysis was separately studied with results in Figure 4. Triplicate experiments were done on the L- and D-Phe adenylate anhydrides with standard deviations shown in Table I. The lines were drawn from least-squares slopes.

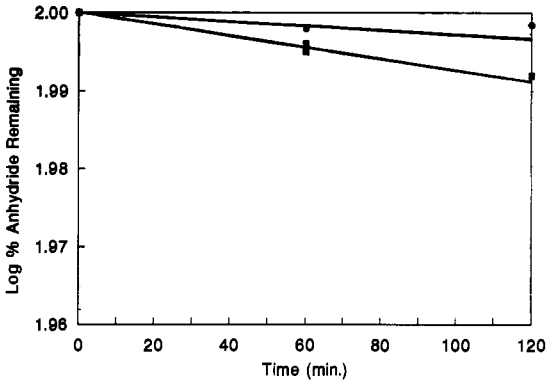

FIGURE 4: Plot of log percent original L- and D-Phe AMP anhydride remaining based on the amount of free AMP appearing at pH 5.0 and 22 °C as a function of time. The AMP is principally formed from hydrolysis of the anhydride. The amount formed from hydrolysis of the product ester is insignificant. Triplicate experiments were done on the D-(■) and L-Phe adenylate anhydrides (■) with results shown in Table I. The lines are drawn from least-squares slopes.

drolysis, as well as the disappearance of the anhydride and the appearance of ester. The results in Figure 3 are for total disappearance of anhydride (hydrolysis plus ester formation).

On the basis of the rate of disappearance of anhydride, the rate constants for combined hydrolysis plus ester formation are given in the third column of Table I. The rate constants for hydrolysis are given in column 4. In Figure 3, it can be seen that the L-anhydride is disappearing much faster than the D. The average rate constant for L disappearance is 15.4×10^{-4} min^{-1} but 2.7×10^{-4} min^{-1} for the D isomer. As can be seen from Figure 4 and Table I, the rate constants for hydrolysis are very low in both cases, 0.79×10^{-4} min^{-1} for L and 1.53×10^{-4} min^{-1} for D. However, because the rate constant for ester formation is so low in the case of the D isomer, the hydrolysis becomes a significant factor in calculating the efficiency whereas in the case of the L isomer the rate constant for ester formation is so much greater than hydrolysis, the amount of hydrolysis is not so significant.

Clearly the differences in efficiencies in Figure 2 are principally due to differences in the rate of ester formation rather than to differences in rates of hydrolysis of the anhydrides. The average rate constant for L-ester formation is 14.7×10^{-4} min^{-1} and for D is 1.1×10^{-4} min^{-1}. One other possibility is that the product esters are hydrolyzing at different rates. From the beginning experiments, it was obvious that the product esters were both hydrolyzing too slowly to be a significant contribution to the difference in the transfer experiments, which never went over 120 min. Overnight sampling was required for the esters, and the hydrolysis rate constants were found to be 1.1×10^{-5} min^{-1} for the L and 0.9×10^{-5} min^{-1} for the D. These are not significantly different from each other and do not contribute substantially to the difference in transfer of the D and L isomers.

Because there is a possibility that this transfer with Phe is an exception rather than the rule, we carried out similar experiments with D- and L-Leu adenylate anhydride at pH 6.1 using the same experimental protocol as with Phe. The L-Leu

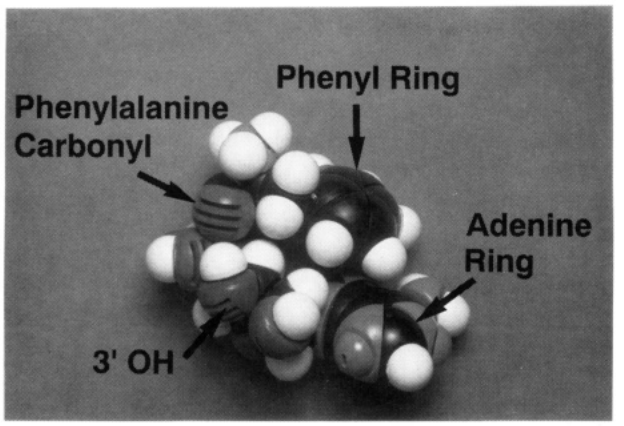

FIGURE 5: Corey–Pauling model of L-Phe AMP anhydride showing a possible phenyl ring association with the adenine ring as suggested by ^{1}H NMR studies (Lacey et al., 1985) and showing the proximity of the amino acid carbonyl to the 3′-OH group of the ribose.

transfer proceeded with 50% efficiency and the D-Leu with 25%. So the preferential transfer of hydrophobic L-amino acids may be the rule, but only further experiment can determine that.

There seems little doubt that this difference between the transfer of D- and L-Phe is real. Fortunately, there also appears to be a possible explanation for this difference. We have previously documented the generality of intramolecular interactions between hydrophobic amino acids and the adenine ring in the aminoacyl adenylates (Lacey et al., 1986). More importantly, we had shown that intramolecular interactions between Ac-L-Phe and the adenine ring are stronger than those between Ac-D-Phe and the adenine ring of aminoacyl adenylates (Lacey et al., 1985).

The most obvious explanation for the preferential transfer of L-Phe is then that associations between the amino acid side chain and the adenine ring are stabilizing the transition intermediate more so with the L isomer than with the D isomer. In Figure 5 is a Corey–Pauling molecular model of L-Phe adenylate anhydride. As can be seen, the carbonyl group of L-Phe can be brought very near to the 3′-OH group of the ribose. One possible intermediate would involve transfer of the proton from the 3′-OH to the carbonyl oxygen with a subsequent transfer of the carbonyl carbon to the 3′-oxygen, yielding a tetrahedral intermediate. We suggest that this transition intermediate is stabilized by the association of the phenyl ring with the adenine ring with greater association and stabilization in the case of the L isomer. A similar model with D-Phe also allows the carbonyl group to approach the 3′-OH group; however, NMR results have shown the D-Phe associates much less strongly with the adenine ring (Lacey et al., 1985). If the amino acid side chain–adenine interaction is responsible for the higher rate and efficiency of transfer of L-Phe, then such a preference may be observed for all hydrophobic amino acids. Because a number of amino acids (Phe, Leu, Ile, Val, and Met) have adenylic acid as their middle, and most important, anticodonic letter, the results here also have some overtones relating to the question of the origin of the genetic code.

From the model in Figure 5, it would appear that the transfer from the phosphate to the ribose would occur at the 3′-position most easily. Preliminary experiments suggest that this is the case even though esterifications with aminoacyl imidazolides seem to take place predominantly at the 2′-position (Lacey et al., 1990b).

We have recently proposed a model as to how purine mononucleotides could serve to preferentially catalyze the synthesis of L-amino acid peptides via a bis(2′,3′-aminoacyl) intermediate (Lacey et al., 1989, 1990a). That model requires esterification to take place at the 2′-position as with the aminoacyl imidazolides (Lacey et al., 1990b). Because the present anhydride to ester transfer seems to be taking place at the 3′-position, it is not presently clear how the present results relate to the model (Lacey et al., 1989, 1990a) if indeed it does relate to it.

Also, it must be emphasized that while the present results show a decided preference for transfer of L-Phe, it must be true that 5′-AMP with an L-ribose sugar should show a preference for D-Phe. Consequently, what these experiments possibly explain is the biological chiral coupling between the D-ribose nucleotides and L-amino acids. While that finding itself is of importance, we must now explain why nucleotides with D-ribose arose as the preferred ones.

Because the transfer process requires the rigorous exclusion of CO_2, in order for it to have been significant early in evolution it would have required either an atmosphere low in CO_2 or absorption of the compound onto a surface to remove it from contact with CO_2.

In an earlier report (Lacey et al., 1988), we showed another difference between D- and L-amino acids in the differential distribution between the 2′- and 3′-positions in aminoacyl esters of 5′-AMP. All L-amino acids distribute ~65% 3′ and 35% 2′ while D-amino acids and carboxylic acids distribute to the 3′-position generally less than the L-amino acids and inversely as a function of the hydrophobicity of the amino acid. We suggested this consistency of L-amino acids in favoring the 3′-position was responsible for the origin of that step in protein synthesis which requires the amino acid to be in the 3′-position to form the peptide bond (Hecht, 1977; Sprinzl & Cramer 1979; Wagner et al., 1982; Taiji et al., 1985).

In related studies, Profy and Usher (1984) and Usher and Needels (1984) did report an excess of L isomer in 2′-OH esterification products of IpI and poly(A) with *tert*-butoxycarbonyl- or dinitrobenzoyl-blocked amino acids. However, when free amino acids were used, the esterification proceeded with an excess of D isomer reacting, the D excess increasing as the hydrophobicity of the amino acid increased. Similarly, Weber (1987) showed an excess of D-serine in the products of 2′,3′-esterification of adenosine 5′-*O*-methylphosphate. So, to our knowledge, the present study is the first to report a significant favoring of a free L-amino acid in the rate and efficiency of formation of an ester of a ribonucleotide.

In one additional study, we have found that the 2′,3′-bis-(diesters) of 5′-AMP are readily formed from preexising monoester and that diester formation from preexisting Ac-L-Phe AMP monoester proceeds about 2.5 times faster than from preexisting Ac-D-Phe AMP monoester (Lacey et al., 1990c). Although no reaction is known to take place with arginine, Yarus and his co-workers (Yarus, 1988) have shown a stereoselective association of L-arginine with RNA in the tetrahymena group I splicing reaction. Furthermore, Gabbay and Kleinman (1970) reported stereoselective interaction of a number of L-amino acid derivatives with nucleic acids. Similarly, Reuben (1978) reported a preference for L-Trp over D-Trp in the association with 5′-AMP.

The present work and others cited constitute mounting evidence for a chiral coupling between L-amino acids and D-sugars. We must, however, still explain the origin of the preference for D-sugars, in other words, the origin of chirality.

Finally, we need to comment on previous studies of the hydrolysis of Phe AMP anhydride and Ac-Phe AMP anhydride (Lacey et al., 1984). Those studies were made by using

a UV method for disappearance of the anhydride. At the time, we had no knowledge of the ester formation from anhydride. It now appears that the results for Phe AMP anhydride in that report involved mostly ester formation. The rate constant for anhydride disappearance at pH 5 was 1.78×10^{-3} min^{-1} and in the present report is 1.54×10^{-3} min^{-1}. The present report must be regarded as the more thorough and definitive one. Consequently, we can now conclude that the rate of hydrolysis of these anhydrides in the absence of CO_2 is really surprisingly slow.

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Reversible Denaturation of the Gene V Protein of Bacteriophage f1†

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ABSTRACT: The guanidine hydrochloride (GuHCl)-induced denaturation of the gene V protein of bacteriophage f1 has been studied, using the chemical reactivity of a cysteine residue that is buried in the folded protein and the circular dichroism (CD) at 211 and 229 nm as measures of the fraction of polypeptide chains in the folded form. It is found that this dimeric protein unfolds in a single cooperative transition from a folded dimer to two unfolded monomers. A folded, monomeric form of the gene V protein was not detected at equilibrium. The kinetics of unfolding of the gene V protein in 3 M GuHCl and the refolding in 2 M GuHCl are also consistent with a transition between a folded dimer and two unfolded monomers. The GuHCl concentration dependence of the rates of folding and unfolding suggests that the transition state for folding is near the folded conformation.

It has been known for many years that denatured proteins can spontaneously fold into their native conformations (Anfinsen, 1973). Despite intensive study, however, neither the mechanism of protein folding nor the details of the interactions that determine the conformations and stabilities of folded proteins are well understood (Ghelis & Yon, 1982; Creighton, 1984). Moreover, only a small number of proteins with known three-dimensional structures have been extensively analyzed in folding and stability studies. Most of these proteins are small monomeric proteins, and they have either largely helical or mixed α-helix and β-sheet secondary structures (Matthews, B. W., 1987; Alber, 1989). To be certain of the generality of observations on protein folding, studies on proteins in different structural classes and with varying states of oligomerization would be useful. We have therefore set out to develop a new model system for studying the process of protein folding

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