Enthalpy of Nucleotides Binding to Myosin†

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ABSTRACT: The enthalpies of binding adenosine 5′-diphosphate (ADP) and 5′-adenylyl imidodiphosphate [AMP-P(NH)P] to rabbit skeletal myosin have been measured in Pipes and Tris buffers at pH 7.8 and 15 °C. For ADP the enthalpy of binding was exothermic, whereas the enthalpy of binding AMP-P(NH)P, a nonhydrolyzable ATP analogue, was small and endothermic. For the reaction of ATP and myosin, the development of enthalpy was resolved into two phases: a fast endothermic phase, which is the summation of binding and hydrolysis, and a slow exothermic phase, which is associated with product-release steps. These results are discussed in terms of their implications for energy transduction.

Transduction of the chemical energy of ATP hydrolysis to mechanical energy in the actomyosin ATPase system is not understood at the molecular level. Several years ago it was suggested that the transfer of free energy between ATP and myosin occurred in the binding process (Morales, 1975; Morales & Botts, 1956). From considerations of the overall standard free energy it was possible to determine that the standard free energy of ATP hydrolysis on the enzyme surface was small. Indeed, kinetic analysis of the actomyosin ATPase has shown that the equilibrium constant for hydrolysis on the enzyme surface is only 9 at 22 °C (Bagshaw & Trentham, 1973).

Energy transduction would be possible if a portion of the free energy of this strong binding interaction, $K \simeq 10^{11}$ M^{-1} (Wolcott & Boyer, 1974; Goody et al., 1977), were retained in the myosin structure to be released as mechanical work when the product complex engages, i.e., forms a cross bridge, with actin.

For most chemical processes our understanding is sharpened if we know how the free energy is partitioned between enthalpy and entropy. In the study of energy transduction in muscle this is of particular interest as these thermodynamic parameters will allow us to use our intuition to postulate new or reaffirm old transduction mechanisms.

In earlier calorimetric experiments, Yamada et al. (1973) measured the enthalpy of ATP hydrolysis by heavy meromyosin and noted that heat production lagged behing hydrolysis as measured by phosphate release. These workers suggested that an energy-storing product complex was formed, although no assessment of enthalpy development at early times was made. Coupled with Morales' earlier suggestion, this prompted us to look closely at the enthalpy of binding, the first step in the myosin ATPase mechanism. In the present study we have made measurements on the enthalpy of ADP and AMP-P(NH)P[1] binding to myosin. The binding enthalpy for ADP is moderately exothermic and typical of enthalpies of inhibitors binding to enzymes. In contrast, the enthalpy of binding AMP-P(NH)P, a nonhydrolyzable ATP analogue, to myosin is endothermic. We have also followed the rate of enthalpy production for the reaction of ATP and myosin as a function of time. Heat production is resolved into a fast phase, which includes binding and hydrolysis, and a slow phase, which is associated with product release. Some implications for energy transduction are presented.

Materials and Methods

The chemicals used in all experiments were of reagent grade and used without purification unless noted. Disodium salts of ATP and ADP were obtained from Sigma Chemical Co. AMP-P(NH)P was obtained from ICN and P-L Biochemicals. The purity of these nucleotides was assessed by chromatography on PEI–cellulose plates. ATP and ADP were greater than 98% pure, and AMP-P(NH)P was greater than 90% pure. AMP deaminase was obtained from Sigma Chemical Co. All solutions were prepared by using glass-distilled water.

Myosin. Rabbit skeletal myosin was prepared and characterized as described earlier (Goodno & Swenson, 1975). Myosin was stored at 4 °C as a pellet precipitated from ammonium sulfate. The purity of the myosin was assumed to be 90–95% and was routinely examined by $NaDodSO_4$–polyacrylamide electrophoresis. The myosin concentration in the solutions was determined by ultraviolet ($E_{280}^{1\%} = 5.5$) and by the biuret assay which was standardized with bovine serum albumin. The calcium and EDTA ATPase activities of myosin were assayed under the conditions of Kielley & Bradley (1956) and Kielley et al. (1956). Specific activities were 0.90 ± 0.02 (SEM) and 3.04 ± 0.08 (SEM) μmol of P_i min^{-1} mg^{-1} for calcium and EDTA assays, respectively, for 20 preparations of myosin.

Myosin solutions were routinely tested for myokinase activity by a coupled assay using AMP deaminase. Most myosin samples showed no myokinase activity but Ap_5A was used in the solution to assure that no spurious heat would be developed in the myosin–ADP binding experiments.

Solutions for Calorimetry. Myosin stock solutions were prepared by homogenizing a predetermined weight of myosin pellet into 20 mM Tris or Pipes buffer which contained 500 mM KCl and 10 mM $MgCl_2$ at pH 7.8. This solution was extensively dialyzed vs. this same buffer until the pH values agreed to ±0.005 pH unit. The concentration of the myosin stock ranged from 5 to 9 mg/mL, which corresponds to a site concentration of 21–38 μM, assuming two sites per 470 000 daltons (Godfrey & Harrington, 1970). Ap_5A was added to a concentration of 0.02 mM from a stock solution in the same buffer for which the pH was adjusted to match the outer dialysate.

Stock nucleotide solutions were prepared in the same buffer,

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[1] Abbreviations used: AMP-P(NH)P, 5′-adenylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; $NaDodSO_4$, sodium dodecyl sulfate; PEI, poly(ethylenimine).

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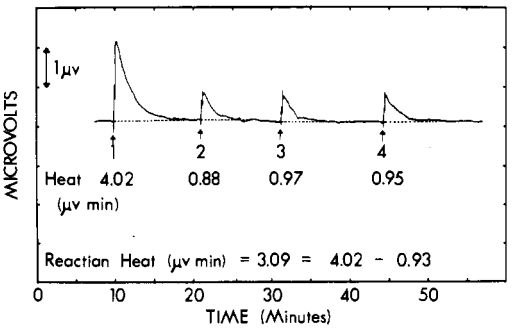

FIGURE 1: Typical calorimetric trace for the binding of ADP to myosin. The calorimeter contained 36.8 nmol of nucleotide binding sites (myosin) and 100 nmol of ADP. The reaction was initiated at the arrow (1) by rotation of the calorimeter. Rotations 2–4 provide values for the mechanical heat. The three mechanical heats were averaged and subtracted from rotation 1 to give the reaction heat for the experiment. Heats of dilution for myosin and nucleotide (generally less than 0.2 mJ) were determined in a similar procedure and were combined with this data and the calibration factor, 0.444 mJ (μV min) to yield the enthalpy of binding.

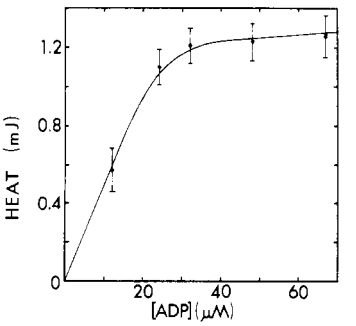

FIGURE 2: Typical measurement of the heat of reaction between ADP and myosin as a function of total ADP concentration in Pipes buffer. The myosin concentration was equivalent to 24.5×10^{-6} M nucleotide binding sites in 1.5 mL. The solid line was calculated by assuming two sites per myosin molecule and an association constant of 1.2×10^6 M^{-1} (Kodama & Woledge, 1976). The enthalpy for this experiment was calculated to be 35 kJ mol^{-1}.

and the pH values were adjusted to ±0.005 pH units of the outer dialysate. Concentrations were calculated from the measured ultraviolet absorption of adenine ($\epsilon_m = 15.4 \times 10^3$ cm^{-1} M^{-1}) and were 200–250 μM. Lower nucleotide concentrations were achieved by dilution of this stock solution. Ap_5A was added to these solutions to a concentration of 0.02 mM.

Calorimeter. The calorimeter was of the twin heat-conduction design and was constructed in our laboratory. The calorimetric vessels were made of gold and typically mixed 1.0 mL (enzyme) and 0.5 mL (nucleotide) of reactant solutions. Measured heats ranged from 0 to ±1200 mJ. Calibration of the calorimeter was accomplished by using the neutralization of Tris by hydrochloric acid (ΔH_i = 48.13 kJ mol^{-1} at 15 °C).

The calorimeter inherently measures the rate of heat production, and thus the quantity of heat is proportional to the integral of the voltage–time trace. In operation, the voltage output from the calorimeter was fed to an interface which performed the analogue to digital conversion, and the digitized output was stored in a Monroe 1860 programmable calculator. When data collection was complete, the computer performed the integration and output the heat equivalent in microvolt minutes. This quantity multiplied by the calibration factor gave the measured heat in millijoules.

In a typical experiment the reactant solutions were loaded into the two compartments of the calorimeter cell. After allowing 1–2 h for temperature equilibration, which is indicated by a "flat" base line (<0.5-μV change in 10 min), we initiated the reaction by rotating the calorimeter body. The integral of the voltage–time curve represents the reaction heat plus a mechanical heat which arises from the viscous heating in the solution when the calorimeter is rotated. This mechanical heat is measured in three or more subsequent rotations. The average value for the mechanical heat is subtracted from the combined mechanical and reaction heats of the first rotation to give the net reaction heat. This is illustrated in Figure 1. Base lines are indicated by the dashed lines.

For rapid processes, such as the binding of ADP illustrated in Figure 1, there is a high rate of heat production for a short time. Signal output is of course the convolution of the true signal with the impulse response function of the calorimeter which is an exponential with a relaxation rate of 1.0 min^{-1}. The observed signal is then a spike, followed by a decay to base line with the relaxation rate of the calorimeter.

The interaction of ATP with myosin produces a more complex function for the rate of heat production vs. time. Kinetic studies have shown that the binding and hydrolysis steps are fast and that product release is slow. For our consideration the following simplified kinetic scheme (Lymm & Taylor, 1970, 1971; Taylor et al., 1970; Taylor, 1977) was used.

$$M + ATP \underset{1}{\overset{\text{fast}}{\rightleftharpoons}} M{\cdot}ATP \underset{2}{\overset{\text{fast}}{\rightleftharpoons}} M^*{\cdot}ADP{\cdot}P \underset{3}{\overset{\text{slow}}{\rightleftharpoons}} M + ADP + P_i$$

We have taken advantage of these differences in rates to resolve enthalpy production for this interaction into two phases: a fast phase, which is the sum of enthalpies of binding and hydrolysis steps, and a slow phase, which corresponds to the product-release step.

Results

All the enthalpies reported have been corrected for the small heats of dilution of myosin and nucleotide. Heat of dilution is usually assessed to solute–solute interactions in the solution; however, in our work it includes any heat changes from mixing nucleotide or myosin and buffer. It is in a sense a control which enables correction for slight pH mismatches of solution as well as for any solute–solute interactions.

ADP–Myosin. Calorimetric data for the binding of ADP to myosin were collected at 15 °C in Tris and Pipes buffers for six different myosin preparations. A typical binding curve for this interaction in Pipes buffer is shown in Figure 2. The solid line is calculated for an association constant of 1.2×10^6 M^{-1} (Kodama & Woledge, 1976), assuming two identical binding sites. Enthalpy values for ADP binding to myosin from eight separate series of experiments in Tris and Pipes buffers, each of which gave a binding curve, were averaged and are presented in row one, columns two and three, of Table I with the calculated standard error. In column four is the enthalpy of binding ADP to myosin obtained by averaging the measured enthalpies in Tris and Pipes after correcting for proton release. Although in principle we could have determined the number of protons released from the enthalpy measurements in two buffers, we have corrected our results using the value measured by Kodama & Woledge (1976) as we feel it is more accurate. Our measured enthalpies were consistent with 0.1–0.2 mol of proton release per mol of nucleotide bound.

AMP-P(NH)P–Myosin. The mixing of AMP-P(NH)P with myosin for nucleotide concentrations ranging from 20 to 75 μM typically produces a calorimetric output wherein the measured heat of the first rotation is only slightly smaller than

Table I: Measured Enthalpies for the Interaction of Nucleotides with Myosin at 15 °C

	ΔH (kJ mol^{-1} of nucleotide) in Tris	ΔH (kJ mol^{-1} of nucleotide) in Pipes	ΔH (kJ mol^{-1} of nucleotide)
	Binding of Nucleotides		
ADP	−39 ± 4	−31 ± 4	−29 ± 3[a]
AMP-P(NH)P	−5 ± 4	0 ± 4	+3 ± 4[a]
	Binding and Hydrolysis		
ATP		+19 ± 4	+21 ± 4[b]

[a] Enthalpy (mean ± SE) is obtained by correcting the measured enthalpies in Tris and Pipes for the release of 0.2 mol of proton per mol of nucleotide bound (Kodama & Woledge, 1976) and averaging the result. The heats of ionization used for Tris and Pipes were respectively 48.13 and 11.45 kJ mol^{-1} [b] Enthalpy (mean ± SE) is obtained by correcting the measured enthalpy in Pipes buffer as described in footnote *a*. It is not corrected for the equilibrium concentration of M·ATP (10–15%) which does not contribute to the fast phase. For this reason, and also because of our procedure for separating the fast and slow phases, this value should be regarded as the minimum endothermic enthalpy for binding and hydrolysis.

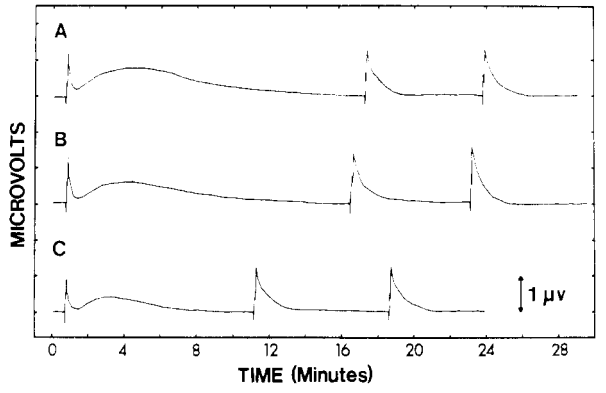

FIGURE 3: Enthalpy development as a function of time for the reaction of ATP and myosin. (A) Myosin (17.0 μM) (sites) and ATP (40 μM) in 1.5 mL; (B) myosin (17.0 μM) (sites) and ATP (26 μM); (C) myosin (17.0 μM) (sites) and ATP (13.3 μM).

the mechanical heat. After correction for nucleotide dilution and myosin dilution, the heat is endothermic but its magnitude is too small to define a meaningful binding isotherm. The best value for the interaction of AMP-P(NH)P and myosin from 20 experiments at near-saturating levels of nucleotide in Pipes buffer is presented in Table I. Also included in the table is the enthalpy of binding corrected for the release of 0.2 mol of proton per mol of nucleotide.

ATP–Myosin. The rate of heat production as a function of time for approximately a single turnover (0.8) of the hydrolysis of ATP by myosin in Pipes buffer is shown in Figure 3C. Traces for mole ratios of nucleotide to myosin site of 2.4 and 1.5 are shown in parts A and B of Figure 3. As expected, heat production occurs in two phases. The first is rapid and includes the summed enthalpic contributions of binding and hydrolysis. It is not clear why this endothermic phase was not observed by Yamada et al. (1973), but it is possible that it was masked by nucleotide dilution heats. There is a suggestion of its presence in their observations in that the corrected curves for ATP intersect time zero below the origin. The second phase, which overlaps the first, results from a low rate of heat production and is associated with product-release steps. The shape of the second phase is markedly influenced by the fact that the rate of product release and the relaxation rate of the calorimeter are similar.

Knowledge of the relaxation rate of the calorimeter and the

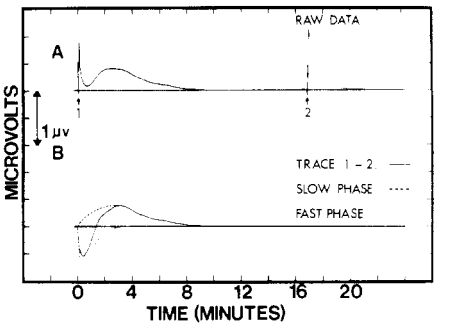

FIGURE 4: Resolution of the slow and fast phase of enthalpy production. (A) Calorimetric output from reaction of 17 μM myosin sites and 13 μM ATP in a 1.5-mL solution (1) and mechanical heat (2). (B) (—) Corrected output trace obtained by subtraction of the mechanical heat; (---) calculated output for the early part of the slow phase by using as the amplitude the maximum amplitude at ~3 min; (···) calculated output for the fast phase obtained by subtracting the slow phase from the corrected output.

rates for the processes involved makes it possible to computer fit the signal as a function of time and obtain amplitudes and decay times for the two phases. It did not seem warranted for the following reason. The sensitivity limit of our calorimeter is being approached in these measurements. This limit is determined by small variations in the mixing efficiency of the solutions in the sample cells which give rise to variations in the time constant of the calorimeter and electrical noise from the thermopiles. These fluctuations lead to significant amplitude variation in the fast phase which makes it unlikely that a unique fit would be achieved.

Resolution of the heats of binding and hydrolysis from the heat of product release was performed by a graphical procedure which is illustrated in Figure 4. The mechanical heat (signal 2) is subtracted from signal 1 in Figure 4A to give the solid curve in Figure 4B which is the rate of enthalpy production vs. time for the reactions of interest convolved with the impulse response of the calorimeter. Kinetic studies show that the rapid phase is complete in a fraction of a second and thus the signal would decay from the maximum amplitude with the time constant of the calorimeter. For the purpose of this discussion, let us assume that a reasonable enthalpy for binding and hydrolysis is 50 kJ mol^{-1} (endothermic). This number would be smaller if the respective enthalpies of binding and hydrolysis were of opposite sign and larger if they were of the same sign. For a sample of the size used in these measurements, the signal amplitude expected is approximately 2 μV. If this signal then decays with the time constant of the calorimeter (1.0 min^{-1}), the amplitude will be reduced to 10% after 2 min and less than 3% after 4 min. Thus, the contribution of the fast phase to the observed signal is negligible after 2 or 3 min.

The true signal for the slow phase, which is at a maximum in a fraction of a second, is known from kinetic studies to be a single exponential with a rate constant of about 0.9 min^{-1} for the mole ratios of enzyme to substrate used in these experiments (Sleep, unpublished experiments). When this signal is convolved with the impulse response of the calorimeter, the observed signal amplitude is expected to increase to a maximum and then decay according to the kinetics of product release convolved with the relaxation rate of the calorimeter. For our graphical procedure we have used the observed maximum as the maximum for the slow phase and constructed the signal for the slow phase at early times from the relaxation rate of the calorimeter. Subtraction of this curve from the observed signal yielded a single exponential for the fast phase. Since the true signal for the slow phase decreases with time

commencing at the completion of the fast phase, the observed maximum is too small, and thus by this procedure we obtain the minimum endothermic enthalpy for binding plus hydrolysis. The procedure, though lacking in sophistication, is in our opinion more than adequate to show that the enthalpy of binding plus hydrolysis is endothermic. In Table I we present the results on the enthalpy of binding plus hydrolysis for the ATP–myosin reaction. The enthalpy presented was calculated from 10 experiments wherein the mole ratio of nucleotide to myosin ranged from 0.5 to 1.2 and has been corrected for release of 0.2 mol of proton per mol of nucleotide bound to myosin.

Discussion

Our results indicate a dramatic difference in the enthalpy of binding ADP and AMP-P(NH)P, the nonhydrolyzable ATP analogue, to myosin. The enthalpy of binding ADP is moderately exothermic, −29 kJ mol^{-1} of nucleotide bound. It should be noted that there is a discrepancy between our results and those of other workers (Kodama & Woledge, 1976). We have obtained smaller negative enthalpies. Banerjee & Morkin (1978) obtained 42 kJ mol^{-1} (after correcting their value for 0.2 mol of proton released to Tris per mol of nucleotide bound) for the binding of ADP to cardiac myosin. Kodama and Woledge, however, obtained a value of 66 kJ mol^{-1} of nucleotide bound to rabbit skeletal myosin. A small part of this discrepancy, approximately 5 kJ mol^{-1}, is due to the fact that we assumed two sites for ADP binding per mol of myosin whereas these authors obtained a value of 1.74 sites by a fitting procedure. In spite of this unexplained difference, it is clear that the enthalpy of ADP binding to myosin is exothermic and markedly different than the enthalpy of binding AMP-P(NH)P to myosin measured in these studies. It is this difference which we wish to discuss.

Morales (1975) had earlier suggested that binding was an important step in the transfer of free energy between subsystems in the myosin ATPase system from a consideration of the kinetics and overall thermodynamics. For ADP and AMP-P(NH)P the binding constants range from 1×10^6 to 2.5×10^6 (Kodama & Woledge, 1976; Schliselfeld, 1974) and reveal little of how the system may transduce energy. Insight into the transduction mechanism may be improved by a knowledge of the partition of the free energy between enthalpy and entropy.

For these two nucleotides our results show that the binding of ADP to myosin, which has the smaller standard free energy, has the larger negative enthalpy. The enthalpy, −29 kJ mol^{-1} of nucleotide bound, is typical for the binding of enzyme inhibitor complexes. This decrease in enthalpy is expected for the interaction between a substrate and its binding site on the enzyme surface. In sharp contrast to this behavior is the measured enthalpy for binding of AMP-P(NH)P, a close structural and electronic analogue of ATP, the substrate for this enzyme. It seems clear that AMP-P(NH)P should fit the binding site on myosin at least as well as ADP as it more closely approximates ATP even though it does not duplicate all the effects of ATP in the actomyosin system (Greene & Eisenberg, 1978). Thus, the observed near-zero enthalpy of binding AMP-P(NH)P is likely to be the result of the coupling of binding, which has a negative enthalpy with some endothermic process.

Fluorescent changes are observed when nucleotides bind to myosin (Werber et al., 1972). For ATP binding the fluorescent change is maximal and contains contributions from binding and hydrolysis. Binding of AMP-P(NH)P and ADP produces respectively 60 and 30% of the fluorescence change associated with the interaction of ATP. Thus, a significant fluorescent change can be associated with the binding process. The fluorescent change is consistent with a substantial conformational change(s) of unknown nature. When considered with these spectroscopic observations, it seems likely that the endothermic enthalpy arises because AMP-P(NH)P binding is coupled to a conformational change and that ADP binding produces only small local conformational changes.

The rate of enthalpy production for the interaction of ATP with myosin is complex in that hydrolysis occurs simultaneously with binding. Although the combined processes are endothermic, it is *impossible* to dissect out the contribution for the binding of ATP for comparison with the enthalpies of binding of ADP and AMP-P(NH)P. Some interesting observations can be made, however. Hydrolysis of ATP on the enzyme surface is endothermic (Taylor, 1977) so it could in fact simply dominate a small exothermic heat of ATP binding. This seems unlikely as the enthalpy of binding ATP, if exothermic, would likely be larger than that of ADP binding as previously noted. The binding constant is 10^5 larger than that of ADP although the effect need not be only enthalpic. On the basis of this information, the sum of binding and hydrolysis should be a small or negative enthalpy rather than the observed positive value. We then suggest that the enthalpy for binding ATP is likely to be similar to that for binding AMP-P(NH)P in that is is likely to be coupled to a conformational change. Coupling of a strong binding process to an unfavorable conformational process can be visualized as an effective way to store this energy in the enzyme structure for use in later kinetic steps. Even though the mechanism cannot be clearly defined, it is clear from the kinetics of heat production in this study and in an earlier study by Yamada et al. (1973) that such storage does occur.

Summary

The observed near-zero enthalpy for the binding of AMP-P(NH)P to myosin when compared with the moderately negative enthalpy for ADP is interpreted to mean that the binding of this ATP analogue is coupled to an energy-requiring conformational change. The endothermic enthalpy measured for the combined processes, ATP binding and hydrolysis, is suggestive of a similar coupling for ATP binding to myosin.

References

Bagshaw, C. R., & Trentham, D. R. (1973) *Biochem. J. 133*, 323–328.

Banerjee, S. K., & Morkin, E. (1978) *Biophys. J. 21*, 15a.

Godfrey, J. E., & Harrington, W. F. (1970) *Biochemistry 9*, 894–908.

Goodno, C. C., & Swenson, C. A. (1975) *Biochemistry 14*, 867–872.

Goody, R. S., Hofman, W., & Mannherz, H. G. (1977) *Eur. J. Biochem. 78*, 317–324.

Greene, L. E., & Eisenberg, E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 54–58.

Kielley, W. W., & Bradley, L. B. (1956) *J. Biol. Chem. 218*, 653–659.

Kielley, W. W., Kalckar, H. M., & Bradley, L. B. (1956) *J. Biol. Chem. 219*, 95–101.

Kodama, T., & Woledge, R. C. (1976) *J. Biol. Chem. 251*, 7499–7503.

Lymm, R. W., & Taylor, E. W. (1970) *Biochemistry 9*, 2975–2983.

Lymm, R. W., & Taylor, E. W. (1971) *Biochemistry 10*, 4617–4624.

Morales, M. F. (1975) *J. Supramol. Struct. 3*, 105–111.

Morales, M. F., & Botts, J. (1956) *Currents in Biochemical Research* (Green, D. E., Ed.) Interscience, New York.
Schliselfeld, L. H. (1974) *J. Biol. Chem. 249*, 4985–4989.
Taylor, E. W. (1977) *Biochemistry 16*, 732–740.
Taylor, E., Lymm, R. W., & Moll, G. (1970) *Biochemistry 9*, 2984–2991.
Werber, M. M., Szent-Gyorgyi, A. G., & Fasman, G. D. (1972) *Biochemistry 11*, 2872–2883.
Wolcott, R. G., & Boyer, P. D. (1974) *Biochem. Biophys. Res. Commun. 57*, 709–716.
Yamada, T., Shimizu, H., & Suga, H. (1973) *Biochim. Biophys. Acta 305*, 642–653.

Biosynthesis of Slaframine, (1*S*,6*S*,8a*S*)-1-Acetoxy-6-aminooctahydroindolizine, a Parasympathomimetic Alkaloid of Fungal Origin. 3. Origin of the Pyrrolidine Ring†

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ABSTRACT: The phytopathogen *Rhizoctonia leguminicola* has previously been shown to incorporate pipecolic acid into the piperidine alkaloids 1-acetoxy-6-aminooctahydroindolizine (slaframine) and 3,4,5-trihydroxyoctahydro-1-pyrindine. In the experiments described here, resting cultures of *R. leguminicola* were incubated with [1-^{14}C]- and [2-^{14}C]malonic acid and with [1-^{14}C]- and [2-^{2}H]acetic acid. Both acids were incorporated into the ring systems of both alkaloids. Mass spectrometric analysis of ^{2}H-enriched slaframine showed that the label resides in the five-membered ring and that the methyl carbon of acetate is joined to the carboxyl carbon of pipecolate. A pipecolate-dependent decarboxylation of [1-^{14}C]malonate was demonstrated in cell-free extracts of *R. leguminicola*. The results account for previously unattributed carbons in the two alkaloids and suggest the formation of an eight-carbon intermediate common to both alkaloids by acylation of malonate with pipecolic acid.

Slaframine (**6**) is unusual among the piperidine alkaloids derived from lysine in that the carboxyl carbon of the amino acid is preserved in the final product (Guengerich et al., 1973b). Figure 1 illustrates some possible routes from lysine to slaframine within the context of our prior knowledge. Experiments described in the first two papers of this series (Guengerich et al., 1973b; Guengerich & Broquist, 1973) demonstrated that the phytopathogen *Rhizoctonia leguminicola* incorporates lysine into slaframine via pipecolic acid (**1**). It was shown that certain potential bicyclic precursors of slaframine [1-keto- (**4**), 1-hydroxy- (**5**), and 1,6-dihydroxyoctahydroindolizine] could be converted to slaframine by *R. leguminicola*, but no information was gained on compounds intervening between pipecolic acid and the bicyclic compounds.

With the demonstration that acetate as acetyl coenzyme A (acetyl-CoA) is the immediate precursor of the 1-acetoxy substituent of slaframine (Guengerich et al., 1973b), all carbons in the alkaloid were accounted for, except for the two completing the pyrrolidine ring.

Because *R. leguminicola* can convert pipecolic acid to a second piperidine alkaloid with different ring fusion [3,4,5-trihydroxyoctahydro-1-pyrindine, **7** (Guengerich et al., 1973a)], again with retention of the pipecolate carboxyl, it was attractive to postulate (Clevenstine & Broquist, 1976) addition of two carbons to the pipecolate carboxyl carbon to give an intermediate (**2**) capable of cyclization to either product; likely routes from **2** to the alkaloids via known intermediates are shown in Figure 1.

The structures of the two alkaloids would readily accommodate condensations of the C_2 donor with the pipecolyl fragment either at the acid or the aldehyde oxidation states, but Guengerich provided evidence (Guengerich et al., 1973b) favoring condensation at the carboxyl level. These considerations implicated "pipecolylacetate" or a related species (**2**) as the fugitive common intermediate and prompted us to test acetate and malonate as potential C_2 donors. The data acquired enabled us to rule out some of the possible alternatives offered in Figure 1.

Materials and Methods

Isotopic Compounds. Disodium [1-^{14}C]- and [2-^{14}C]-malonate and sodium [1-^{14}C]acetate were purchased from New England Nuclear or Amersham/Searle. Partition chromatography (Ramsey, 1961) showed that the malonate from Amersham/Searle was contaminated with a small quantity of acetate, which was removed by the same technique. DL-(*R*)-[^{3}H]Pipecolic acid (180 mCi/mmol) came from stocks prepared by Guengerich from material synthesized at New England Nuclear by catalytic hydrogenation of picolinic acid with tritium gas (see Guengerich et al., 1973b).

Deuterioacetic acid (Gold Label, 99.5% $C^2H_3COO^2H$) was purchased from Aldrich Chemical Co.

Cultivation of Rhizoctonia leguminicola and Isolation of Alkaloids. The organism (ATCC 26280) was maintained on slants of filtered red clover hay infusion (1000 mL of water per 100 g of dry chopped hay) hardened with 1.5% Bacto-Agar (Difco). Inoculum for experiments was prepared by transferring a tuft of mycelium from a slant to the surface of 240 mL of sterile hay infusion in a 1000-mL Roux bottle. After 2 weeks growth at room temperature, the mycelial mat was blended in 150 mL of sterile distilled water, and approximately

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