A new one-pot synthesis was designed to prepare benzoyl-AMP under anhydrous conditions in N,N-dimethylformamide. Reaction of benzoic acid with N,N′-carbonyldiimidazole and subsequently with 5′-adenosyl monophosphate gave the mixed anhydride in 76% isolated yield. The structure of benzoyl-AMP was confirmed by mass spectroscopy and 1H-, 31P-, and 13C-NMR. The purity of the preparation was greater than 98% as indicated by 31P- and 13C-NMR. Purified aryl-aldehyde oxidoreductase was incubated in NMR tubes together with either carboxy-13C-benzoyl-AMP or carboxy-13C-benzoic acid to demonstrate that benzoyl-AMP is an active intermediate during the enzymatic activation of benzoic acid to benzaldehyde.

Mixed 5′-adenosic acid-carbonyl anhydrides are common intermediates formed during the enzymatic activation of carboxylic acids by ATP. Such carbonyl-AMP intermediates are involved in: fatty acyl-CoA synthesis (1), the linkage of biotin to apocarboxylase to form holocarboxylase (2), the metabolism of valproic acid in liver (3), amino acid activation in tRNA acylation by Pseudomonas (8), and during fatty aldehyde formation in bioluminescence in Photobacterium (9). Facile enzymatic reductions of carboxylic acids to aldehydes by aryl-aldehyde oxidoreductases are typically ATP- and NADPH-dependent (1) and involve putative acyl-AMP intermediates (10, 11).

Several approaches for the synthesis of acyl-AMP mixed anhydrides have been described (12–17). Earlier aqueous solvent-based syntheses generally gave low product yields, presenting significant difficulties during reaction work up, and gave acyl-AMP derivatives that were poorly characterized. Most commonly, the condensation of AMP and carboxylic acids has involved initial coupling of carboxylic acids with N,N′-dicyclohexylcarbodiimide (DCCD) (13) in alkaline medium. Ethylchloroformate was used as a carboxylic acid coupling reagent in the synthesis of relatively impure, N-protected aminoacyl-adenylates (14). Racemic isobutyl chloroformate was later used instead to afford acyl-adenylates that were characterized by elemental analysis and IR and low-field 1H-NMR spectroscopies. Isobutyl chloroformate is not widely used because syntheses are complicated by the presence of an unwanted chiral center in the coupling reagent.

The availability of highly pure and well characterized carboxyl-AMP derivatives is essential when attempting to clarify enzyme mechanisms involving such intermediates. For example, a poorly defined synthetic acyl-AMP derivative led to incorrect conclusions that biotin activation was not involved in holocarboxylase synthesis from apocarboxylase (18, 19).

N,N′-Carbonyldiimidazole has been used to selectively activate carbonyl groups, even in the presence of potentially reactive functional groups like phenols (20). In an effort to elucidate the nature of intermediates involved in the enzymatic reduction of carboxylic acids to aldehydes (7), we sought to develop a simpler, nonaqueous-based synthesis of carboxyl-AMP by using N,N′-carbonyldiimidazole as a coupling reagent. We report the first complete structure elucidation of benzoyl-AMP. Furthermore, carboxy-13C-benzoyl-AMP was synthesized and used in a 13C-NMR study of Nocardia aryl-aldehyde oxidoreductase to confirm the involvement of benzoyl-AMP in the enzymatic reduction of benzoic acid to benzaldehyde.

**EXPERIMENTAL PROCEDURES**

**Spectroscopy Methods—** 13C- (90.6 MHz) and 31P-NMR (145.7 MHz) spectra were obtained with a Bruker WM 360 spectrometer. 1H, heteroatom multiple bond correlation, and heteroatom multiple quantum correlation (HMQC) (21, 22) NMR spectra were obtained with a Bruker AMX 600 spectrometer. All samples were dissolved in D2O/DCl (5% DCl), and 2-13C-acetic acid was used as the internal standard for 13C (δ = 20.08) NMR. Neat phosphoric acid was used as the external standard for 31P-NMR. NMR chemical shifts (δ) are reported in parts per million (ppm), and the coupling constants (J values) are in Hertz (Hz). NMR abbreviations are s = singlets, d = doublets, dd = doublets of doublets, t = triplets, and m = multiplets. High resolution fast atom bombardment MS was performed at the University of Iowa, high resolution mass spectrometry core facility using a VG analytical ZAB instrument with polyethylene glycol 600/Nal/3-nitrobenzyl alcohol as the carrier. Ultraviolet spectra for benzoyl-AMP and AMP were determined in triplicate in water solutions using a Shimadzu Model UV-2101PC scanning spectrophotometer.

**Thin Layer Chromatography—** Thin layer chromatography (TLC) was carried out on a 0.25-mm thick layers of silica gel GF254 developed with solvent system A, benzene:acetonitrile:water (80:20 v/v/v) containing 1% formic acid, or solvent system B, acetic acid:water:butanol (2:3:5 v/v/v). With solvent system A, benzoic acid and benzoylimidazole gave Rf values of 0.58 and 0.28, respectively. With solvent system B, benzoyl-AMP and AMP gave Rf values of 0.50 and 0.25, respectively. Developed TLC plates were visualized under 254-nm UV light or by spraying plates with Von’s reagent (24 g of ammonium molydbdate, 1 g of ceric ammonium sulfate, and 31 ml of H2SO4 in 500 ml of water).

**Synthesis of Benzoyl-AMP—** Benzoyl-AMP was prepared by the reaction sequence outlined in Fig. 2. In a typical reaction, 36 mg of benzoic acid (0.30 mmol) was reacted with 51 mg of N,N′-carbonyldiimidazole (0.31 mmol) in 10 ml of anhydrous DMP (Aldrich).
under N₂ at room temperature for 2 h, at which time the reaction was essentially quantitative. Then, 105 mg of 5′-AMP (0.30 mmol, free acid, dried over P₂O₅, Sigma), 20 ml of anhydrous DMF, and 0.5% pyridine (Aldrich, Sure/Seal container under N₂) in 0.1 ml of DMF were added to the reaction mixture, which was stirred at room temperature for an additional 72 h. TLC analysis showed that the reaction was complete with only one spot at Rf 0.50 for benzoyl-AMP (estimated 90% yield).

**Purification of Benzoyl-AMP—**All chromatographic procedures were carried out at 4 °C. The reaction mixture was diluted with 200 ml of 50 mM sodium acetate buffer, pH 5.2, and loaded onto a DE52 ion exchange column (2.6 x 20 cm, chloride form). Benzoyl-AMP was eluted with a gradient of NaCl from 0 to 150 mM in the starting buffer (700 ml). The fractions (8.5 ml each) containing benzoyl-AMP (fraction 55–76) were combined and lyophilized. The lyophillized preparation was then dissolved in 15 ml of 1 mM HCl and passed over a Bio-Gel P-2 column (5 x 100 cm). The benzoyl-AMP-containing fractions were pooled and lyophilized to give 760 mg of dry powder containing 108 mg of sodium benzoyl-AMP (0.208 mmol, 76% yield). The final preparation was stored at −20 °C. This product was completely characterized by MS and 1H, 13C, and 31P-NMR spectral analyses.

The content of benzoyl-AMP in the lyophilized powder was determined both by enzyme analysis and by hydrolysis of benzoyl-AMP and UV quantitation of the resulting AMP product. For enzymatic quantitation, the reaction mixture contained 0.15 mM NADPH and 0.01 unit of purified carboxylic acid oxidoreductase in 50 mM Tris-HCl, pH 7.5. The amount of benzoyl-AMP was determined by measuring the ΔA₂₆₀ nm as NADPH was oxidized to NADP⁺. For hydrolysis, 10-mg samples of benzoyl-AMP preparations were dissolved in 100 ml of 0.1 N NaOH and hydrolyzed at room temperature for 5 h. The AMP content of the reaction mixture was determined by comparing optical densities of the hydrolyzed solutions at 259 nm to absorbances for standards of AMP in NaOH.

**Nocardia Aryl-aldehyde Oxidoreductase—**The enzyme was produced with 13C-carboxy-labeled benzoyl-AMP by the same method. The purified product, benzoyl-AMP (0.216 mmol, 72% yield), was characterized by TLC, MS, and with 13C- and 31P-NMR.

**Results**

**Benzoyl-AMP** was prepared in a simple, two-step sequence. In the first step, based on TLC analysis, the reaction between benzoic acid and N,N-carbonyldiimidazole was quantitative within 2 h at room temperature. The coupling of AMP to the benzoyl group in the second step was enhanced by the inclusion of 0.5% pyridine. After 72 h, the reaction mixture contained one product, benzoyl-AMP (Rf = 0.5) and small amounts of AMP (Rf = 0.25). The final product gave a single TLC spot at Rf = 0.50, and the purity was confirmed to be greater than 98% by NMR spectroscopy.

Benzoyl-AMP gave a high resolution fast atom bombardment MS molecular ion at 474.0778 for C₁₇H₁₈N₅O₈Na (calculated, 474.0787). The UV spectrum showed λmax values at 237 nm (ε = 15,900) and 260 nm (ε = 12,900). These absorptions were comparable with that for AMP, which shows λmax at 260 nm (ε = 13,200). Proton-carbon NMR spectral correlations of benzoyl-AMP were established by HMQC (22), and these assignments were further confirmed by heteronatom multiple bond correlation spectroscopy (21) as shown in Table I. For example, the proton signal for H-1’ at δ = 6.01 is attached to C-1’ (by HMQC) and is two-bond correlated with C-2’, three-bond correlated with C-4, 8, 3’, and 4’ to unambiguously confirm this assignment. Benzoyl-AMP preparations were estimated to be >98% pure because no AMP containing impurities could be detected by 13C- and 31P-NMR. The signal for the carbonyl-carbon of benzoyl-AMP was a doublet at δ 162.25 (J = 8.4 Hz) because of coupling with the 31P nucleus.

**Carboxy-13C-benzoyl-AMP** was prepared by the same method. Carboxy-13C-benzoyl-AMP gave a carboxy carbon signal at δ 162.25 (d, J = 8.4 Hz) by 13C-NMR and gave a phosphorus signal at δ = −6.52 ppm (d, J = 8.4 Hz) by 31P-NMR, clearly demonstrating the anhydride coupling between the 13C and 31P nuclei. The complete assignments of signals and 13C-31P coupling results were essential to show that the carboxylphosphate anhydride exists, versus other undesirable isomers in which, for example, the benzoyl group was linked to AMP through either the amino group, or a ribose oxygen atom of the adenyl moiety.

To be detected as a free metabolite such as valproyl-AMP (3), carbonyl-AMP derivatives require reasonable aqueous stability. For a 0.5 mmol solution of benzoyl-AMP in 50 mM, Tris-HCl, pH 7.5, at 25 °C, the pseudo first-order rate constant was determined to be 1.92 x 10⁻² ± 3.3 x 10⁻⁴ h⁻¹. This indicates that benzoyl-AMP is sufficiently stable for use as a substrate in enzyme kinetic and mechanism studies. Lyophillized benzoyl-AMP is stable for at least 6 months at −20 °C.

NMR Spectroscopy was used to obtain direct structural information for the involvement of benzoyl-AMP in the aryl-aldehyde oxidoreductase catalyzed reduction of benzoic acid to benzaldehyde. To enhance NMR spectral analyses of putative intermediates and products, we utilized both 13C-carboxy-enriched benzoic acid and benzoyl-AMP as substrates for purified compounds. All 13C-NMR spectral signals in enzyme reactions were obtained by accumulations of 760 pulses.

**Fig. 1. Proposed chemical mechanism for benzoic acid reduction by aryl-aldehyde oxidoreductase.**

**Fig. 2. Synthesis of benzoyl-AMP.** The reagents were: N,N′-carbonyldiimidazole, anhydrous DMF, 2 h at room temperature (a), anhydrous 5′-AMP, 0.5% pyridine, 72 h at room temperature (b).
aryl-aldehyde oxidoreductase, ATP-, and NADPH-dependent reductions conducted in NMR tubes. Use of isotopically enriched substrates permitted the ready acquisition of spectra in enzyme reactions. Three types of enzyme reactions were examined, each containing in addition to enzyme: ATP and hydroxylamine together with labeled benzoic acid (Fig. 3A), NADPH with labeled benzoyl-AMP (Fig. 3B), and NADPH and ATP with labeled benzoic acid (Fig. 3C). After 40-min incubations at room temperature, all were analyzed to give the \(^{13}\)C-NMR spectral results shown in Fig. 3.

In Fig. 3, spectrum A contains carbonyl signals at both \(\delta = 176.4\) and \(\delta = 169\) for unutilized benzoic acid substrate and the benzoyl-hydroxamate derivative, showing that the benzoyl-AMP intermediate is formed during the enzymatic reduction reaction. Spectrum B shows a single signal at \(\delta = 202.8\) for the carbonyl carbon atom of benzaldehyde, the product formed when benzoyl-AMP is reduced by the enzyme with NADPH. Spectrum C shows signals at \(\delta = 176.4\) and \(\delta = 200.8\) for residual, unreacted benzoic acid substrate and product benzaldehyde, respectively.

**DISCUSSION**

\(\text{N,N'}\)-Carbonyldiimidazole is a useful, general carboxylic acid activating reagent because it selectively reacts with carboxylic acids even in the presence of hydroxy or phenolic functional groups (20). In this study, carbonyldiimidazole was used to obtain unambiguous coupling of AMP and benzoic acid to give benzoyl-AMP in high yield and facilitated the chromatographic purification of the product. Benzoyl-AMP was fully characterized by spectral analysis for the first time. All proton and carbon signals were clearly assigned by the use of standard proton-carbon correlation spectroscopies and by carbon-phosphorus couplings. In previous studies, Armstrong et al. (15) used the P-O-C infrared stretch between 1040–1090 cm\(^{-1}\) as partial spectral evidence for the existence of a mixed acyl-phosphoanhydride. Low field \(^{1}H\)-NMR spectroscopy showed H-8 as a broad doublet, a spectral observation that cannot be explained for a single acyl-AMP isomer. The precise elaboration of all carbon NMR signals for benzoyl-AMP (Table I) was an essential step prior to the use of \(^{13}\)C-carbonyl-enriched benzoic acid or benzoyl-AMP as substrates for NMR experiments designed to identify the in situ formation of intermediates in the aryl-aldehyde oxidoreductase reaction. Furthermore, the half-life of 36 h for dilute solutions of benzoyl-AMP in Tris-HCl, pH 7.5, at 25 °C ensured its stability as a substrate in mechanism studies.

### Table I

| Carbon | \(\delta_{C,\text{NMR}} - \text{ppm}\) | \(\delta_{P, \text{NMR}} - \text{ppm}\) | HMBC\(^a\) |
|--------|-----------------|-----------------|---------|
| 2      | 150.2           | 8.09 (1H, s)    | C6, C4  |
| 4      | 146.8           |                 |         |
| 5      | 116.5           |                 |         |
| 6      | 152.9           |                 |         |
| 8      | 137.7           | 8.29 (1H, s)    | C6, C4  |
| 1\*    | 85.0            | 6.01 (1H, d, \(J = 5.6\)) | C6, C4, C3, C5, C4, |
| 2\*    | 71.5            | 4.9 (1H, t, \(J = 5.5\)) | C1, C3  |
| 3\*    | 68.5            | 4.64 (1H, dd, \(J = 5.1, 4.2\)) | C1, C3, C5  |
| 4\*    | 81.8 (d, \(J = 6.6\)) | 4.48–4.51 (2H, m) | C2, C1 |
| 5\*    | 64.6 (d, \(J = 6.2\)) | 4.48–4.51 (2H, m) | C2, C1  |
| 6\*    | 126.2 (d, \(J = 7.7\)) | 7.16 (2H, t, \(J = 8.2, 1.1\)) | C6, C7, C2, C5  |
| 1'     | 126.3           | 7.2 (2H, t, \(J = 7.8\)) | C6, C4, C1 |
| 4'     | 132.2           | 7.47 (1H, t, \(J = 7.5\)) | C1 |
| 7'     | 162.2 (d, \(J = 8.1\)) |                 |         |

\(a\) Split by coupling with \(^{31}\)P.

\(b\) Heteratom multiple bond correlation signals are listed by order of intensity.

**FIG. 3.** \(^{13}\)C-NMR study of the mechanism of benzoic acid reduction catalyzed by *Nocardia ardia* aryl-aldehyde oxidoreductase. NMR spectra were obtained using incubations containing: carboxy-\(^{13}\)C-benzoic acid + ATP + \(\text{NH}_2\text{OH}\) (A); carboxy-\(^{2}\)C-benzoyl-AMP + NADPH (B); and, carboxy-\(^{13}\)C-benzoic acid + ATP + NADPH (C).

Previous studies which implicated carbonyl-AMP intermediates during enzymatic carboxylic acid reduction (1–4, 10) relied primarily on the use of radiolabeled precursors and chromatographic (TLC, gas chromatography) analyses to identify metabolites. With an enzyme from *Neurospora crassa*, Gross (10) trapped \(^{13}\)C-labeled benzoyl-AMP as a hydroxamate derivative that was identified by TLC. \(^{15}\)C-labeled benzaldehyde was chromatographically identified as the product obtained when \(^{13}\)COOH-labeled benzoate was used as substrate. Although Kato et al. (24) implicated benzoyl-AMP as an intermediate in benzoic acid reduction by an enzyme from *Nocardia asteroides*, it was not characterized.

NMR analyses of the enzymatic reductions of benzoic acid with ATP only (Fig. 3A), with benzoyl-AMP plus NADPH (Fig. 3B), or with ATP plus NADPH (Fig. 3C) provided direct evidence for the involvement of benzoyl-AMP in the reduction reaction. Attempts to measure benzoyl-AMP directly as an intermediate in the conversion of benzoic acid to benzaldehyde were unsuccessful. Because benzoyl-AMP is likely a transient intermediate present only in low steady-state concentrations, others have indirectly measured benzoyl-AMP formed in similar enzyme reactions by use of hydroxylamine. Hydroxamate derivatives can only be formed with acyl-adenylates and not with benzoic acid. In previous studies, such hydroxamates have been detected with thin layer chromatography (10, 23). When hydroxylamine was used as a “trapping” reagent in our experiment (Fig. 3A), the signal for the carbonyl group of unutilized \(^{13}\)C-labeled benzoic acid appeared at 176.4 ppm, whereas the hydroxamate derivative appeared at 169 ppm. No reaction was observed when benzoic acid, ATP, and the carboxylic acid reductase were reacted together in control incubations.

The precise kinetic mechanism by which benzoic acid is
reduced to benzaldehyde remains to be established. Whether benzoyl-AMP is actually released from the enzyme active site during catalysis is not known. Benzoyl-AMP, has a significantly lower (67-fold) apparent $K_m$ (9.66 ± 0.71 μM), a higher (1.3-fold) $V_{\text{max}}$ (7.50 ± 0.18 μmol min$^{-1}$ mg$^{-1}$) than those for benzoic acid (7). These results indicate that steady-state levels of benzoyl-AMP are low during the course of the reduction of benzoic acid to benzaldehyde. The results of this study, together with our previous work in which benzaldehyde was isolated and characterized spectrally, clearly demonstrate that the mechanism for benzoic acid reduction to benzaldehyde by purified *Nocardia* sp. NRRL 5646 aryl-aldehyde oxidoreductase, involves benzoyl-AMP as an intermediate.

Acknowledgment—We are grateful to John Snyder for help with NMR instruments.

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J. Biol. Chem. 1998, 273:34230-34233.
doi: 10.1074/jbc.273.51.34230

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