Bacterial Acetone Carboxylase is a Manganese-Dependent Metalloenzyme†

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INTERNET: ensigns@cc.usu.edu
Abbreviations:

EPR, electron paramagnetic resonance
ICP/MS, inductively coupled plasma atomic emission mass spectrometry
PEP, phosphoenolpyruvate
Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase
ABSTRACT

Bacterial acetone carboxylase catalyzes the ATP-dependent carboxylation of acetone to acetoacetate with the concomitant production of AMP and 2 inorganic phosphates. The importance of manganese in *Rhodobacter capsulatus* acetone carboxylase has been established through a combination of physiological, biochemical, and spectroscopic studies. Depletion of manganese from the *R. capsulatus* growth medium resulted in inhibition of acetone-dependent but not malate-dependent cell growth. Under normal growth conditions (0.5 µM Mn$^{2+}$ in medium), growth with acetone as the carbon source resulted in a four-fold increase in intracellular protein-bound manganese over malate-grown cells, and the appearance of a Mn$^{2+}$ EPR signal centered at $g = 2$ that was absent in malate-grown cells. Acetone carboxylase purified from cells grown with 50 µM Mn$^{2+}$ had a 1.6-fold higher specific activity and 1.9-fold higher Mn content than cells grown with 0.5 µM Mn$^{2+}$, consistently yielding a stoichiometry of 1.9 Mn / α$\beta_{2}$$\gamma_{2}$ multimer, or 0.95 Mn / αβ$\gamma$ protomer. Mn in acetone carboxylase was tightly bound and not removed upon dialysis against various metal ion chelators. The addition of acetone to malate-grown cells grown in medium depleted of Mn resulted in the high level synthesis of acetone carboxylase (15-20% soluble protein) which, upon purification, exhibited 7% of the activity and 6% of the Mn content of the enzyme purified from acetone-grown cells. EPR analysis of purified acetone carboxylase indicates the presence of a mononuclear Mn$^{2+}$ center, with possible spin coupling of two mononuclear sites. The addition of Mg ·ATP or Mg ·AMP resulted in EPR spectral changes while the addition of acetone, CO$_{2}$, inorganic phosphate, and acetoacetate did not perturb the EPR. These studies demonstrate that manganese is essential for acetone carboxylation and suggest a role for Mn in nucleotide binding and activation.
INTRODUCTION

Acetone is a toxic molecule that is produced biologically by the fermentative metabolism of certain anaerobic bacteria and from ketone body breakdown in mammals (1,2). In addition to producing acetone, mammals and microorganisms also metabolize acetone, producing acetoacetate or acetol (1-hydroxyacetone) from the carboxylation or hydroxylation, respectively, of acetone (3-12). Despite more than 60 years of research on the subject, and direct evidence for enzymatic conversion of acetone to acetoacetate and acetol, the physiological significance of acetone metabolism in mammals remains unclear (2,13). The role of acetone metabolism in bacteria is more clearly defined, in that a variety of aerobic and anaerobic bacteria are able to grow using acetone as their primary source of carbon and energy (14).

Despite some early evidence suggesting that acetol is an intermediate in aerobic acetone metabolism by some bacteria (4,6,15), it now appears that carboxylation of acetone to acetoacetate is the primary, if not only, reaction by which both aerobic and anaerobic bacteria initiate acetone catabolism (14). Acetone carboxylation by bacteria has been studied extensively in cell suspensions and extracts of diverse bacteria (3,5,7,9,16-22). Recently, acetone carboxylases (E.C. 6.4.1.6) have been purified and characterized from two bacteria: *Xanthobacter autotrophicus*, an aerobic alpha proteobacterium, and *Rhodobacter capsulatus*, a purple nonsulfur photosynthetic bacterium (23,24). The acetone carboxylases from the two bacteria had nearly indistinguishable biochemical properties, consisting of three subunits of 85, 79, and 20 kDa arranged in an $\alpha_2\beta_2\gamma_2$ quaternary structures (23,24). The purified enzymes did not contain nor require biotin. Purified preparations of the enzymes contained significant quantities of manganese, zinc, and iron, with manganese being present in the highest quantity (1.3 – 1.5 mol Mn/mol $\alpha_2\beta_2\gamma_2$ protomer) (23,24). The enzymes also exhibited identical catalytic
and kinetic properties, catalyzing the ATP-dependent carboxylation of acetone to acetoacetate according to Equation 1 (23,24):

\[
\text{CH}_3\text{COCH}_3 + \text{CO}_2 + \text{ATP} \rightarrow \text{CH}_3\text{COCH}_2\text{COO}^- + \text{H}^+ + \text{AMP} + 2\text{Pi} \quad (\text{Eq. 1})
\]

The formation of AMP and 2 inorganic phosphates as ATP hydrolysis products is unprecedented for all other carboxylase reactions known.

For both bacteria, the genes encoding the acetone carboxylase subunits are arranged in operons consisting of the genes \(\text{acxABC}\), which encode the beta, alpha, and gamma subunits of the enzyme, respectively (24). While the amino acid sequences of the three subunits share very high identity with each other (70 – 84%), they are not homologous to any other carboxylases, including Rubisco and the biotin-dependent carboxylases (24). In fact, the only enzymes that share significant identity with acetone carboxylase are hydantoinases, which show 15-30% identity with the \(\alpha\) and \(\beta\) subunits of acetone carboxylase (24). The gamma subunit of acetone carboxylase does not share significant identity with any known enzyme.

Together, the biochemical, kinetic and molecular properties of acetone carboxylase suggest that it represents a fundamentally new class of carboxylase enzyme. Acetone carboxylation is thermodynamically unfavorable, and, not surprisingly, the reverse reaction, acetoacate decarboxylation, occurs spontaneously in aqueous solution. One of the first enzymes to be studied in detail mechanistically was acetoacetate decarboxylase, which accelerates the spontaneous decomposition of acetoacetate to acetone and \(\text{CO}_2\) (25). Acetoacetate decarboxylase does not share homology with acetone carboxylase, does not require a nucleotide cofactor, and is not capable of catalyzing the carboxylation of acetone. Thus, acetone carboxylation and acetoacetate decarboxylation are not freely reversible reactions and require distinctive strategies of catalysis.
Given the lack of precedents for the acetone carboxylase reaction, we have begun to examine the cofactor requirements of the enzyme in order to gain insights into the catalytic mechanism. Particularly intriguing is the presence of significant quantities of manganese in the purified enzyme. Manganese is a cofactor for a number of enzymes, but rarely remains tightly bound and associated with enzymes during purification. In the present study, the possible role of manganese in acetone carboxylase has been probed by a combination of physiological, biochemical, and spectroscopic studies. The results demonstrate that manganese is a required and stoichiometric cofactor of acetone carboxylase, and provide insights into the nature of the manganese center and possible roles of the metal ion in catalysis.
Experimental Procedures

*Media, Growth of Bacteria and Preparation of Cell Extracts*—*Rhodobacter capsulatus* strain B10 (ATCC 33303) (26) was grown phototrophically at 30°C using the media and growth conditions described previously (24), with the following modifications. Concentrated stock solutions of media components, minus divalent metal salts, were passed over a column (5 x 100 cm) of Chelex-100 resin (Bio-Rad) to deplete the media of adventitious metals. All glassware was washed with 1 M HNO₃ prior to use. The appropriate metal salts were then added to complete the media. Where indicated, manganese was either omitted or included in the media formulation. Large scale cultivations were performed in a 45-liter capacity illuminated glass vessel as described previously (24). For small scale cultivation, cultures were grown in a controlled temperature incubator with illumination provided by a combination of 15 watt fluorescent and 60 watt incandescent light bulbs. The cultures were grown in glass tubes (15 ml) sealed with red rubber stoppers (13 x 20 mm sleeve). The carbon sources for growth were acetone (40 mM) and/or malic acid (30 mM), and the source of inoculant was a late-log phase culture of cells grown on the same medium. The optical density of the cultures was determined using a Klett-Summerson Photoelectric Colorimeter. The Klett Colorimeter was standardized relative to a Shimadzu model UV-160 spectrophotometer for the conversion of Klett readings to absorbance values at 600 nm.

Cultures grown in the 45 liter fermenter were harvested after reaching $A_{600}$ values between 2.5 and 4.0 using an A/G Technologies polysulfone membrane cartridge filtration system. Concentrated cell suspensions were pelleted by centrifugation, washed once with 50 mM potassium phosphate buffer (pH 7.2), repelleted, and stored at −80°C. Frozen cell paste was resuspended in an equal volume of buffer A (25mM MOPS pH 7.6, 1mM dithiothreitol, 5%
glycerol) containing DNase I (0.03 mg/ml). Cell suspensions were passed three times through a chilled French pressure cell at 110,000 kPa and 4°C. Cell lysates were clarified by centrifugation (105,000 X g for 1 h at 4°C).

Purification of enzymes and protein characterization. Acetone carboxylase was purified as described previously(24). For purification of acetone carboxylase from manganese-depleted cultures, all buffers were treated with Chelex, and glassware washed with HNO₃ as described above. EDTA (1 mM) was included in the buffers for purification of manganese-depleted acetone carboxylase.

SDS/PAGE (12% T, 2.7% C running gel) was performed in a Mini-Protean II apparatus (Bio-Rad) following the Laemmli procedure(27). Proteins in gels were visualized by staining with Coomassie blue. The standards used were β-galactosidase (116 kDa), Bovine serum albumin (66 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), Restriction endonuclease Bsp981 (25 kDa), β-lactoglobulin (18 kDa), and lysozyme (14 kDa), purchased from Fermentas.

Immunoblot analysis was conducted by electrophoretically transferring proteins from SDS-PAGE gels onto a polyvinylidene difluoride membrane. The membrane was incubated with polyclonal antiserum raised against purified acetone carboxylase. Cross-reacting proteins were visualized using horse radish peroxidase (HRP) conjugated to goat anti-rabbit immunoglobulin G (Promega).

Acetone carboxylase assays. All assay buffers and glassware were depleted of trace metals as described above. Acetone carboxylase activity in purified protein samples was determined using gas chromatography as described previously (24,28).
Spectroscopic Techniques X-band EPR spectra were obtained using a Bruker model ESP 300 spectrometer equipped with an ER 4116 DM dual mode X-band cavity and an Oxford Instruments ESR-900 helium flow cryostat. In all cases, calibrated 4 mm quartz EPR tubes (Wilmad, Buena, NJ) were used. Independently recorded background spectra of the cavity were aligned with and subtracted from experimental spectra. EPR spectra were recorded at a modulation frequency of 100 kHz and amplitude of 1.26 mT (12.6 G) with a sweep rate of 10mT s\(^{-1}\). Spectra were recorded at microwave frequencies of approximately 9.64 GHz, with the precise microwave frequencies noted for individual spectra to ensure exact g-alignment. A standard solution containing CuSO\(_4\) (1mM) and EDTA (10mM) was used for spin quantitation of the metal centers in protein samples.

For EPR and metal analysis of cell lysates, samples were incubated with Chelex-100 for 10 minutes and then passed over a 1.5 X 5 cm column of Sephadex G-25 (PD-10, Amersham Pharmacia) which had 1 cm of chelex overlaid on the resin. The samples for EPR analysis were placed in serum vials and degassed on a vacuum manifold with repeated cycles of evacuation and flushing with argon. The samples were then placed into quartz tubes that had been flushed with argon and were frozen in liquid nitrogen.

For EPR analysis of purified proteins, samples were treated with Chelex-100 followed by centrifugation (1000x g) and decanting to remove the Chelex resin. Nucleotides, acetone, and other molecules were added to appropriate samples, followed by evacuation, flushing and transfer to degassed EPR tubes as described above.

Temperature dependence of EPR Signals. Spectra were recorded at different temperatures using the conditions described above, except that the power was lowered to an
optimal nonsaturating value (0.1 mW). The relative peak heights for each signal were plotted vs. temperature, and the data were fit to Curie law $1/T$ dependence according to Eq. 2:

$$S = \frac{k}{T} + x$$

(Eq. 2)

For this equation, $S$ is the EPR signal intensity, $k$ is an arbitrary constant, $T$ is temperature in Kelvin, and $x$ is a correction factor. The variations in constants were 3.1 to 4.0 for $k$ and 0.11 to 0.35 for $x$.

Analytical procedures: Protein concentrations were determined by means of a modified Biuret assay (29). Metal contents of protein samples were determined by ICP-MS at the Utah State University Veterinary Diagnostics Laboratory. All samples were treated with Chelex-100 as described above prior to performing metal analysis.
RESULTS

Manganese stimulates acetone-dependent growth of R. capsulatus. The acetone carboxylases from R. capsulatus and X. autotrophicus are essentially identical enzymes (24). Both enzymes are induced to high levels (17-25% of soluble proteins) upon addition of acetone to actively-growing cultures, and the induction is not inhibited by the presence of other carbon sources. X. autotrophicus metabolizes acetone under aerobic conditions, while R. capsulatus metabolizes acetone under anaerobic, photoheterotrophic conditions (26). Since acetone is highly volatile, it is easier to control and quantify acetone-dependent growth in the contained, anaerobic conditions used to culture R. capsulatus. For this reason R. capsulatus was chosen for the following physiological studies, and as the source of acetone carboxylase for the subsequent spectroscopic analyses.

The growth medium used to culture R. capsulatus contains 0.5 µM of exogenously added MnCl₂ (26). To determine what effect manganese limitation might have on growth of R. capsulatus, MnCl₂ was excluded from the growth medium, and the components of the growth medium were treated with Chelex-100 to remove adventitious manganese. As shown in Figure 1, R. capsulatus grew poorly with acetone as the carbon source in the media lacking manganese. The doubling time of the manganese-depleted cells during log phase was approximately 19% of the manganese-supplemented cells. It should be noted that a slight amount of manganese (0.75 nM MnCl₂ based on 0.15% v/v inoculant size) was carried over from the manganese-supplemented inoculant used for this experiment. To determine whether manganese carryover contributed to the slow growth on manganese-depleted media, the cells at the 200 minute time point were subcultured again into manganese-depleted media. Similar growth rates were observed, perhaps due to contamination of the media with traces of manganese that were not
removed by Chelex treatment, or limited functionality of a manganese-depleted form of the enzyme.

In contrast to the results described above, there was little, if any, effect of manganese depletion on growth of *R. capsulatus* with malate as the carbon source, both in terms of doubling time and final cell density (Figure 1). Additionally, manganese-depleted, malate-grown cells could be subcultured repeatedly on media lacking manganese with no apparent effect on growth rates or yields. Thus, manganese is required for optimal acetone-dependent, but not malate-dependent, growth of *R. capsulatus*.

*Acetone carboxylase is synthesized in manganese-depleted, malate-grown cells.* *R. capsulatus* cells were grown with malate as the carbon source for several generations in the absence of manganese. As shown in Figure 2, acetone carboxylase was not synthesized under these growth conditions. Upon addition of acetone to the culture, acetone carboxylase was induced and synthesized at very high levels, despite the presence of malate and absence of added manganese in the growth medium. Thus, acetone carboxylase is inducible in *R. capsulatus*, not subject to negative regulation by the absence of manganese, and not subject to catabolite repression. These results are consistent with the previous observation that *R. capsulatus* *acxABC* is under the apparent control of a sigma70-dependent transcriptional activator acxR (24).

*Correlation of acetone-dependent growth with increased intracellular manganese and appearance of a manganese EPR signal.* If acetone carboxylase is indeed a manganese-dependent enzyme, its synthesis may correlate with increased intracellular concentrations of protein-bound manganese. To investigate this, metal analysis was performed on extracts prepared from cells grown with normal concentrations of manganese, either with acetone or malate as the carbon source. Before performing metal analysis, the extracts were treated with
Chelex resin and desalted by gel filtration chromatography to remove free- and loosely-bound manganese, so the analyses should reflect only manganese bound tightly to cellular macromolecules. The results were as follows: an extract of acetone-grown cells contained 0.45 ppm manganese, while an extract of malate-grown cells with the same concentration of protein (5.5 mg protein/ml for this experiment) contained 0.11 ppm manganese. Thus, growth with acetone as the carbon source resulted in a 4-fold increase in the concentration of intracellular, protein-bound manganese.

Extracts of the cells analyzed for manganese content were also analyzed by EPR spectroscopy in order to determine whether acetone carboxylase synthesis correlates with the appearance of a unique EPR signal that can be attributed to protein-bound manganese. As shown in Figure 3, a six line EPR spectrum centered at about 3500 gauss is visible in the extract from acetone-grown cells but not visible in the extract from malate-grown cells. This spectrum is clearly a manganese (II)-dependent signal, as indicated by the six-fold hyperfine, with average distances of 86 G between adjacent features. As shown in the inset, a spectrum of aqueous MnCl₂ obtained under the same conditions shows very little resolution of hyperfine features due to the anisotropic ligand environment of manganese hexahydrate. Thus, the hyperfine observed in the protein samples cannot originate from free manganese and must result from asymmetric protein bound manganese.

Optimal growth conditions result in stoichiometric manganese in acetone carboxylase. As noted above, the growth medium used to culture *R. capsulatus* contains 0.5 μM supplemental manganese. Based on the high level of synthesis of acetone carboxylase, it is conceivable that manganese becomes limiting if it is a preferred or essential metal ion for the enzyme. The concentration of acetone carboxylase (α₂βγγ multimer) in a culture of *R. capsulatus* grown to an
optical density of 6, and for which acetone carboxylase represents 20% of soluble proteins, can be estimated to be approximately 0.28 µM (based on the culture volume, not the intracellular concentration of protein). This concentration corresponds to 0.56 µM of αβγ protomer, a number slightly higher than the concentration of added manganese. Thus, manganese is potentially limiting under these growth conditions if it is indeed a stoichiometric component of the enzyme.

Table 1 shows the results of manganese supplementation and depletion on the metal contents and specific activities of purified acetone carboxylase preparations. Clearly, there is a positive correlation between manganese availability in the media, manganese content of the purified enzymes, and enzyme specific activity. The addition of 50 µM manganese to the growth medium resulted in the association of an average of 1.9 tightly bound manganese ions per α2β2γ2 multimer, or 0.95 manganese per αβγ protomer. At the same time, the specific activity of the enzyme increased by 1.6-fold over the preparations purified from cells containing only 0.5 µM manganese in the media. Dialysis of acetone carboxylase against buffers containing 5 mM EDTA, 100 mM imidazole, or 25 mM 8-hydroxyquinoline did not result in the loss of manganese from the enzyme, nor did the treatments lower the activity of the enzyme.

The preparation from manganese-depleted cells had very low manganese content and corresponding low specific activity (Table 1). At the same time, the iron content of the preparation was increased dramatically, suggesting that iron may associate with the enzyme in the absence of manganese. The low activity of this enzyme form indicates that iron is not able to substitute effectively for manganese in catalysis. Collectively, the results presented in Table 1 indicate that manganese is a stoichiometric and catalytic cofactor for acetone carboxylase.
EPR analysis of acetone carboxylase. Acetone carboxylase samples prepared from cells grown with optimal manganese were analyzed by EPR spectroscopy in perpendicular mode in order to obtain more information on the nature of the metal center(s). A representative spectrum obtained under optimal power and temperature conditions is shown in Figure 4. Spin integration of the EPR spectrum yielded 1.9 spins per $\alpha_2\beta_2\gamma_2$ multimer, which corresponds to 0.95 spins per $\alpha\beta\gamma$ protomer and 1.0 spins per manganese. A large six-fold Mn$^{2+}$ ($I=5/2$) anisotropic hyperfine centered at $g = 2$ (3360 G,) corresponding to $m_s \pm 1/2$ (30), dominates the spectrum. The average hyperfine splitting of 85 G is suggestive of mononuclear $^{55}$Mn coordinated to oxygen and nitrogen ligands in an octahedral environment (31). The spectral features and splitting are also consistent with a five-coordinate trigonal bipyramidal geometry as reported for manganese-dependent superoxide dismutase (32).

There are two additional interesting features centered at $g=10.2$ (685 G) and $g=4.8$ (1500 G) (Figure 4B). The six-fold hyperfine for the resonance that is centered at $g=10.2$ has an average splitting ($A= 90$ G) consistent with that expected for mononuclear $^{55}$Mn in which no coupling of the paramagnetic center is present. The 11-fold hyperfine splitting for the resonance that is centered at $g=4.8$ has an average splitting of $A=43$ G. This resonance is unique because the hyperfine splitting is approximately one half of what would be expected for a mononuclear center. Hyperfine splitting of these distances have been attributed to exchange-coupled Mn$^{2+}$ ions (31,33-37). This indicates that there may be coupling between two distinct Mn$^{2+}$ centers. Signals around $g=4.8$ that have Mn$^{2+}$ hyperfine have previously been attributed to a “forbidden” $M_s= \pm 2$ transition (38). This transition is often seen in Mn$^{2+}$ complexes which have a large zero field splitting (33,39,40). In the acetone carboxylase spectrum, there is also a shoulder at $g=2.3$ (3000 G) accompanied by a large broad peak centered at $g=1.5$ (4550 G). These resonances have
previously been noted to be forbidden hyperfine transitions which are attributed to zero-field interactions (41). Additionally a shoulder is seen at \( g=3.1 \) (2260 G) as well as a very low field peak at \( g=23.7 \) (291 G).

White et al. have reported five distinguishing characteristics of a mononuclear \( \text{Mn}^{2+} \) binding site as determined for bacteriophage \( \lambda \) phosphoprotein phosphatase (38). These features are: (a) an intense six-fold \(^{55}\text{Mn} \) hyperfine centered around \( g=2 \), (b) a peak at approximately 3000 G that is not part of the \( g=2 \) \(^{55}\text{Mn} \) hyperfine, (c) a broad and relatively featureless peak from 2000-3000 G, (d) a hyperfine split resonance from 1300-2100 G, and (e) a low field transition featuring \(^{55}\text{Mn} \) hyperfine splitting. The spectra shown in Figure 4 exhibit all of these features and a few additional ones.

When analyzed in parallel mode, two very weak features were visible in acetone carboxylase, centered at approximately 1400 and 3000 gauss. The signals were very weak relative to the signals observed in perpendicular mode, and their origins and relevance is uncertain.

Acetone carboxylase is colorless, and the addition of sodium dithionite to the enzyme had no effect on the EPR spectrum (data not shown). This indicates that the manganese is divalent and probably does not have a redox role in acetone carboxylation (42).

The effect of increased temperature on the EPR of acetone carboxylase is shown in Figure 5. Several new features appear at the higher temperature, the most notable being the twin peaks seen at \( g=2.3 \) (2990 G) and \( g=2.2 \) (3077 G). In the 5K spectrum, these distinct peaks can not be seen, but small shoulders are visible at similar \( g \) values. In the 31K spectrum the very low field \( g=23.7 \) (291 G) signal disappears. Also seen at higher temperature is a dip centered at approximately \( g=1.61 \) (4285 G) as well as a very broad upfield feature centered at \( g=1.43 \) (4825 G).
G). Finally, there is a geometric distortion of the broad shoulder seen at \( g = 3.08 \) (2241 G) in the 31K spectrum. These new features seen in the 31 K spectrum may arise from low-lying excited states which are populated as the temperature is increased (31). Hendrich and coworkers have observed a similar phenomenon for spectral features in a manganese-substituted form of the diiron ribonucleotide reductase (43). According to the authors, this increase in signal intensity with increasing temperature is indicative of an antiferromagnetically coupled dimanganese (II) site (43). Thus, the appearance of these new features in acetone carboxylase at higher temperature provides further evidence that the \( g = 2 \) signal may arise from coupled manganese (II) centers. No additional hyperfine splitting can be seen in the higher temperature spectrum. Finally, the \( g = 2 \) signal appears more isotropic at higher temperature.

A more detailed study of the temperature dependence of the EPR signal intensities was conducted in order to probe for possible coupling of manganese centers in the enzyme. The results of this study are presented in the inset to Figure 5. Each of the three signals (\( g = 2, g = 4.8, g = 10.2 \)) decreased in intensity as the temperature was increased, and exhibited Curie Law \( 1/T \) dependent behavior, indicating a ground-state transition. All three signals exhibited similar temperature dependence, but the intensity of \( g = 2 \) species decreased less for a given temperature decrease than the \( g = 4.8 \) and \( g = 10.2 \) species. This difference in signal decay vs. temperature may indicate that the \( g = 2 \) signal arises from a different species, although the difference in decay is not enough to state this conclusively.

Effect of substrates, products, and cofactors on the EPR of acetone carboxylase. The addition of acetone, \( \text{CO}_2 + \text{bicarbonate} \), or acetoacetate to acetone carboxylase samples had no noticeable effect on the EPR properties of the enzyme (spectra not shown). Likewise, the addition of either phosphate or pyrophosphate alone did not invoke significant changes in the
EPR (spectra not shown). The addition of ATP alone (not complexed with magnesium) resulted in some changes in the EPR spectra, while the addition of ADP, AMP or magnesium ion alone provided no noticeable differences (Figures 6 and 7). However, the simultaneous addition of magnesium and nucleoside phosphates resulted in dramatic changes in the EPR of acetone carboxylase (Figures 6 and 7). It should be noted that magnesium ion is required for acetone carboxylation, presumably by complexing with ATP to provide the active form of the nucleotide used (23).

The most profound difference in the EPR is seen upon addition of Mg·AMP, the hydrolysis product of ATP formed during catalysis (see Equation 1). Specifically, a large increase in intensity is seen in the \( g = 2 \) region of the spectrum (Figures 6 and 7). At the same time, a large decrease in the \( g = 10.2 \) and 4.8 hyperfine signals is seen, along with the disappearance of the peaks at \( g = 3.1 \) and 23.7 (Figures 6 and 7).

The addition of ATP alone (without Mg\(^{2+}\)) resulted in the disappearance of the broad peak at \( g = 23.7 \) and the formation of a fourth signal downfield at \( g = 71.5 \) (143 G) which displayed \(^{55}\)Mn hyperfine splitting (Figures 6 and 7). The additional presence of magnesium resulted in more spectral changes, including an increase in intensity of the new \( g = 71.5 \) feature, a shift in position of the broad peak at \( g = 3.1 \) to \( g = 3.5 \), a distortion of the \( g = 4.8 \) feature, and the disappearance of the \( g = 10.2 \) hyperfine.

No changes in the EPR were seen in the presence of ADP alone. Some changes, with similarities to the changes effected by Mg·AMP and Mg·ATP, are apparent in the presence of Mg·ADP. As can be seen in Figures 6 and 7, the changes invoked by Mg·ADP were less dramatic than those seen for the Mg·AMP and Mg·ATP complexes.
DISCUSSION

Our understanding of microbial acetone metabolism was advanced greatly by the purification, characterization, and genetic analysis of acetone carboxylases from two acetone-utilizing bacteria (23,24). These studies revealed that acetone metabolism in both aerobes and anaerobes involves carboxylation, and that the key enzyme of this process, acetone carboxylase, is a conserved enzyme. Acetone carboxylase itself, however, is a largely enigmatic enzyme. The enzyme requires ATP, yet does not use biotin, as do most other ATP-dependent carboxylases (44). Additionally, acetone carboxylation occurs with the unprecedented hydrolysis of two rather than one phosphodiester bonds, in the process forming AMP and inorganic phosphate as products (23,24). Acetone carboxylase does not share sequence homology with the biotin-dependent carboxylases, Rubisco, or enzymes catalyzing reactions bearing similarities to acetone carboxylation, including PEP carboxylase, PEP carboxykinase, and pyruvate kinase (24). Thus, acetone carboxylase appears to be representative of a new class of carboxylases employing a fundamentally different strategy to facilitate substrate carboxylation. Recent work has suggested that a similar type of enzyme may be involved in the metabolism of acetophenone (methylphenylketone) in ethylbenzene-degrading bacteria (45).

The present work provides evidence that acetone carboxylase is a manganese-dependent enzyme. Significantly, manganese remains tightly bound to the enzyme through purification, is not removed by metal chelators, and is present in stoichiometric amounts in enzyme preparations isolated from cells cultured with 50 µM MnCl₂ in the media (Table 1). Thus, manganese is best viewed as an integral cofactor of the enzyme rather than a dissociable metal ion required for catalysis. This is in contrast to the situation for most manganese-dependent enzymes, where manganese is lost during enzyme purification and must be added back to restore enzymatic
activity (46). In general, manganese (II) is viewed as a labile metal ion due to the
thermodynamic instability of its complexes with amino acid side chains, and it’s affinity for
water in forming Mn(H₂O)₆²⁺ (46). It is thus both intriguing and significant that, in acetone
carboxylase, manganese is bound so tightly.

For many enzymes that require a divalent metal ion, in particular nucleoside
triphosphate-dependent enzymes such as pyruvate kinase, creatine kinase, and PEP
carboxykinase, manganese and magnesium ion are able to function interchangeably with little or
no effect on activity (46). In the case of acetone carboxylase, magnesium ion is required as an
additional exogenous cofactor. The evidence suggests that magnesium ion is required for the
formation of the Mg ·ATP complex, the active form of ATP in the assay (23). Since manganese
is tightly bound while magnesium must be added with ATP, the two metal ions are obviously
distinct cofactors with different roles in catalysis. It should be noted as well that acetone
carboxylase activity is also stimulated by the presence of a monovalent ion, e.g. potassium or
ammonium, and potassium ion is included in activity assays for this reason.

The X-Band EPR of the resting enzyme shows spectral features very similar to those
observed for other manganese (II)-dependent or manganese (II)-substituted enzymes
(31,34,37,41,42,47-50). At present, it is unclear from the EPR whether manganese is present in a
mononuclear site, a dinuclear site, or two closely associated mononuclear centers, perhaps
bridged by a permanent or dissociable ligand. The subunit structure of acetone carboxylase is
α₂β₂γ₂, and the optimal manganese stoichiometry is 1.9 Mn/ α₂β₂γ₂ multimer. It is tempting to
speculate that acetone carboxylase is separated into two catalytic αβγ protomeric units, each with
a distinct active site. In this case, each site would be expected to contain a single mononuclear
manganese site based on the observed manganese stoichiometry. It is possible that the active
sites of the two protomeric units are close to each other, resulting in coupling and the observed presence of the dinuclear EPR features. Alternatively, there may be some heterogeneity in enzyme preparations. However, the EPR spectra reported here are identical to those collected from other enzyme preparations, including those with the substoichiometric manganese purified from cells containing 0.5 µM rather than 50 µM manganese (data not shown). Thus, the spectral features are not a consequence of the particular growth or isolation procedures used for a single preparation.

Recent studies of bacteriophage λ protein phosphatase have shown that this enzyme can exist in forms with either a mononuclear or dinuclear manganese center (31). In this case, reconstitution of the apoenzyme with manganese (II) followed by desalting resulted in the formation of the mononuclear enzyme form, which was largely inactive (31). The addition of manganese (II) to this form of the enzyme resulted in increased enzymatic activity and changes in the EPR indicative of conversion of the mononuclear site to an active dinuclear site (31). Thus, there are two manganese sites in bacteriophage λ protein phosphatase: a higher affinity site forming the mononuclear cluster, and a lower affinity site that binds an additional manganese to form the dinuclear site (31). It should be noted that this phosphatase will use other metal ions, including nickel (II) in place of manganese, and that manganese does not associate with the enzyme throughout purification(51,52). Similarly, arginase is inactive as isolated, and is activated by manganese and other metal ions, in the process forming a dinuclear cluster (35). In the case of acetone carboxylase, the enzyme is fully active as isolated from cells grown with 50 µM MnCl₂, contains a full complement of manganese, and the enzyme activity is not stimulated further by addition of exogenous manganese (II) or other divalent metal ions, with the exception of the necessary magnesium. The comparison of bacteriophage λ protein phosphatase, arginase...
and acetone carboxylase reemphasizes the exceptional high affinity for manganese exhibited by acetone carboxylase. Why the enzyme has such high affinity, and the exact nature of the protein-manganese association(s), remain unclear. The three-dimensional structure of acetone carboxylase is currently being solved to shed further lights on these details (53).

Perturbations of the EPR spectra of manganese metalloproteins by the addition of exogenous ligands may be due to changes in the anisotropy of the bound Mn$^{2+}$ and thus are good indicators of changes in the ligand environment (33). As shown in Figures 6 and 7, the addition of the magnesium complexes of adenosine phosphates, in particular Mg ·AMP and Mg ·ATP, result in significant changes in the EPR of acetone carboxylase, while the addition of other relevant compounds do not. These results suggest that manganese is involved in the binding, and perhaps activation, of ATP for catalysis rather than coordinating or activating acetone and/or acetoacetate. At present, it is unclear how ATP hydrolysis supports acetone carboxylation. The carboxylation of acetone is thermodynamically unfavorable ($\Delta G^\circ' = +17.1 \text{ kJ/mol}$), and the hydrolysis of one phosphodiester bond of ATP ($\Delta G^\circ' = -30.5 \text{ kJ/mol}$) would, in theory, provide enough energy to support acetone carboxylation. One attractive model for the role of ATP is in transfer of a phosphoryl group to acetone to stabilize the enol of acetone as phosphoenolacetone. A second phosphate may be involved in activating bicarbonate to carboxyphosphate, although this would need to be done in the absence of biotin. These possible roles of ATP hydrolysis in acetone carboxylation are purely speculative at present. The observation that the magnesium complexes of ATP, ADP, and AMP perturb the coordination environment of manganese in acetone carboxylase is nevertheless significant, in that it suggests manganese plays a role in nucleotide activation rather than some other aspect(s) of catalysis.
In summary, this paper expands our knowledge of the biological roles of manganese by demonstrating the requirement for this metal ion in bacterial acetone metabolism. The role of manganese is distinguished from other manganese metalloproteins by the tight binding of the metal ion to the enzyme, and the apparent inability of other metal ions, including iron, zinc, and magnesium, to substitute for manganese in the reaction.
REFERENCES


FIGURE LEGENDS

Figure 1. Manganese depletion inhibits acetone-dependent growth of \textit{R. capsulatus}. Symbols: Growth with acetone (circles) or malate (triangles) as the reduced carbon source. Closed symbols, 0.5 µM MnCl\textsubscript{2} in medium; Open symbols, Chelex-treated, manganese-deficient medium.

Figure 2. Electrophoretic and immunoblot analysis of acetone carboxylase synthesis in \textit{R. capsulatus}. Panel (A), SDS-PAGE gel; Panel (B), Immunoblot of (A). Lane 1, molecular weight markers (2 µg each); lane 2, purified acetone carboxylase (3 µg) with the α, β, and γ subunits visible from top to bottom of the gel; lane 3, extract of acetone-grown cells from culture containing 0.5 µM MnCl\textsubscript{2} (30 µg); lane 4, extract of malate-grown cells from culture containing 0.5 µM MnCl\textsubscript{2} (30 µg); lane 5, extract from malate-grown cells with acetone present from culture depleted of MnCl\textsubscript{2} (30 µg).

Figure 3. X-band EPR spectra of cell extracts of acetone (a.) and malate (b.) grown \textit{R. capsulatus}. The samples were prepared as described in the methods section. Experimental conditions: Temperature, 5.6 K; modulation frequency, 100 KHz; modulation amplitude, 12.6G; time constant, 81.92 ms; microwave frequency, 9.654 GHz; and microwave power, 2.0 mW. Inset: Spectrum of 0.1 mM MnSO\textsubscript{4} under identical conditions.

Figure 4. X-band EPR spectra of acetone carboxylase at 5.6 K. The concentration of acetone carboxylase was 0.14 mM (A.) Spectrum from 0-6000 G. Inlay in A., spectrum of 0.1 mM MnSO\textsubscript{4} in buffer A. (B.) expanded scale of the 0-3000 G region of the spectrum. The letters a, b, and c show the valleys used to determine the average hyperfine splitting. Experimental
conditions for spectra: Temperature, 5.6 K; modulation frequency, 100 KHz; modulation amplitude, 12.6 G; time constant 81.92 ms; microwave frequency 9.654 GHz; and microwave power, 2.0 mW.

Figure 5. Temperature dependence of acetone carboxylase EPR. (a.) EPR spectrum recorded at 30 K. (b.) EPR spectrum recorded at 5.8 K. With the exception of temperature, the experimental conditions for the two spectra were the same as for Figure 4. The arrows indicate new features resolved in the higher temperature spectrum at $g = 2.3$, $g = 2.2$, and $g = 1.61$. The inset shows the temperature dependence of the $g = 2$ (closed circles), $g = 4.8$ (open circles) and $g = 10.2$ signals at nonsaturating power (0.1 mW). The curve fits were obtained by fitting the data to Curie law $1/T$ dependence as described in the experimental procedures.

Figure 6. Effect of nucleotides on the EPR of acetone carboxylase. The concentration of acetone carboxylase was 0.14 mM for all samples and the experimental conditions were the same as for Figure 4. When present, nucleotides were added to a concentration of 16 mM and MgCl$_2$ was added to 1.6 mM. (a.) no additions (b.) plus MgCl$_2$; (c.) plus AMP; (d.) plus AMP and MgCl$_2$; (e.) plus ADP; (f.) plus ADP and MgCl$_2$. (g.) plus ATP; (h.) plus ATP and MgCl$_2$.

Figure 7. Expanded view of the 0-3000 G region of the spectra shown in Figure 6.
Table 1. Metal contents and activities of acetone carboxylase preparations from *R. capsulatus*.

<table>
<thead>
<tr>
<th>Acetone carboxylase source&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Metal ion / $\alpha_2\beta_2\gamma_2$ multimer (mol/mol)</th>
<th>Specific activity, units ·mg&lt;sup&gt;-1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe</td>
<td>Mn</td>
</tr>
<tr>
<td>Acetone-grown, +0.5 µM MnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>ND</td>
<td>1.01 ± 0.17</td>
</tr>
<tr>
<td>Acetone-grown, +50 µM MnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.03 ± 0.03</td>
<td>1.90 ± 0.05</td>
</tr>
<tr>
<td>Malate-grown with acetone present, no added MnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.45± 0.03</td>
<td>0.12± 0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>The numbers represent the averages from three enzyme preparations prepared from three different batches of cells grown under the indicated conditions. The errors are reported as standard deviations.

<sup>b</sup>For the preparation of manganese-depleted acetone carboxylase, it was necessary to have malate present as a carbon source to support bacterial growth (see Figure 1).
a. Acetone grown

b. Malate grown
Magnetic Field (Gauss)

- a. no additions
- b. + Mg^{2+}
- c. +AMP
- e. + ADP
- f. + ADP + Mg^{2+}
- g. + ATP
- h. + ATP + Mg^{2+}

\[ g = 10.2 \quad g = 4.8 \quad g = 2 \]
Magnetic Field (Gauss)

0 500 1000 1500 2000 2500 3000

a. no additions
b. + Mg^{2+}
c. + AMP
d. + AMP + Mg^{2+}
e. + ADP
f. + ADP + Mg^{2+}
g. + ATP
h. + ATP + Mg^{2+}
Bacterial acetone carboxylase is a manganese-dependent metalloenzyme
Jeffrey M. Boyd, Heather Ellsworth and Scott A. Ensign

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