Identification of Intracellular Carbonic Anhydrase in *Chlamydomonas reinhardtii* which Is Distinct from the Periplasmic Form of the Enzyme

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ABSTRACT

A physiologically significant level of intracellular carbonic anhydrase has been identified in *Chlamydomonas reinhardtii* after lysis of the cell wall-less mutant, cw15, and two intracellular polypeptides have been identified which bind to anti-carbonic anhydrase antisera. The susceptibility of the intracellular activity to sulfonamide carbonic anhydrase inhibitors is more than three orders-of-magnitude less than that of the periplasmic enzyme, indicating that the intracellular activity was distinct from the periplasmic form of the enzyme. When electrophoretically separated cell extracts or chloroplast stromal fractions were probed with either anti-C. *reinhardtii* periplasmic carbonic anhydrase antiserum or anti-spinach carbonic anhydrase antiserum, immunoreactive polypeptides of 45 kilodaltons and 110 kilodaltons were observed with both antisera. The strongly immunoreactive 37 kilodalton polypeptide due to the periplasmic carbonic anhydrase was also observed in lysed cells, but neither the 37 kilodalton nor the 110 kilodalton polypeptides were present in the chloroplast stromal fraction. These studies have identified intracellular carbonic anhydrase activity, and putative intracellular carbonic anhydrase polypeptides in *Chlamydomonas reinhardtii* represented by a 45 kilodalton polypeptide in the chloroplast and a 110 kilodalton form probably in the cytoplasm, which may be associated with an intracellular inorganic carbon concentrating system.

The unicellular green alga, *Chlamydomonas reinhardtii*, has an efficient mechanism for the utilization of CO$_2$ which allows these cells to carry out photosynthesis at optimal rates when only very low concentrations of CO$_2$ are available in the medium ($K_{0.5}$(CO$_2$) < 1 μM) (2, 16, 17, 27). This $K_{0.5}$(CO$_2$) value is considerably less than that required for the optimal utilization of CO$_2$ by the primary carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase ($K_m$(CO$_2$) = 29 μM), isolated from this alga (8). The high affinity for CO$_2$ is apparent only in cells grown with limiting concentrations (air levels, 0.04%) of CO$_2$ (2, 16). Cells grown in the presence of a high concentration of CO$_2$ (5%) exhibit a low affinity for CO$_2$ which is similar to that of ribulose-1,5-bisphosphate carboxylase/oxygenase (2, 16). The efficient utilization of CO$_2$ by algae grown under CO$_2$-limited conditions is attributed to the existence of a C$_i$ concentrating system which results in the intracellular accumulation of C$_i$ to levels higher than can be accounted for by the passive diffusion of CO$_2$ into the cell. In addition, the observed accumulation of C$_i$ is greater than that which would be expected based upon the pH differential between the inside of the cell and the medium (2, 16).

Many studies have shown that CA is an important component of the C$_i$ concentrating system in *C. reinhardtii* and other green algae and cyanobacteria (1). In *C. reinhardtii*, there is a correlation between increased levels of cellular CA and the induction of the C$_i$ concentrating mechanism (5, 10, 34). Furthermore, specific inhibitors of CA both diminish the high apparent cellular affinity for C$_i$ and affect the intracellular accumulation of C$_i$ (2, 14, 17, 19, 24, 27). Several lines of evidence indicate that most of the CA in *C. reinhardtii* is present within the periplasmic space. The majority of the cellular activity can be measured with intact cells (10, 17, 32), is inhibited by plasma membrane-impermeant CA inhibitors (17), is abolished by protease treatment of intact cells (32), and is released into the medium by the cell wall-less mutant, cw15 (5, 10). Several studies indicate that the primary role of the periplasmic CA is to accelerate the extracellular replacement of the nonionic C$_i$ species, CO$_2$, as it diffuses into the cell (6, 16, 17, 27). Thus, the CA reaction is of particular physiological importance at high extracellular pH where HCO$_3^-$ is the predominant form of C$_i$ in solution at equilibrium (16, 17, 27). However, it has also been suggested that CO$_2$ is actively transported (14), and that *C. reinhardtii* may also be able to take up HCO$_3^-$ from the medium under some conditions (31).
In addition to the existence of the periplasmic form of CA, several studies have provided evidence for intracellular forms of CA which may be components of the C\textsubscript{i} concentrating system in \textit{C. reinhardtii} (2, 17, 20, 24, 25). The membrane-permeant CA inhibitor, ethoxzolamide, diminishes the efficiency of C\textsubscript{i} utilization by \textit{C. reinhardtii}, and causes an enhanced accumulation of C\textsubscript{i} intracellularly (2, 17). This is consistent with the presence of an intracellular form of CA which is necessary for rapid conversion of intracellular pools of HCO\textsubscript{3} to CO\textsubscript{2} for the carboxylation of ribulose-1,5-bisphosphate. We have provided evidence that a physiologically important intracellular CA is present even when cells are not adapted to low concentrations of CO\textsubscript{2} (20). Spalding et al. (24) and Tsuzuki et al. (28) have measured an increase in CA activity upon lysis of \textit{C. reinhardtii} cells, and mutants of \textit{C. reinhardtii} have been isolated which appear to be deficient in an intracellular form of CA (21, 24, 25).

In this report, direct evidence is presented for the existence of intracellular CA with properties distinct from the periplasmic form of the enzyme. Antibody probes have been used to identify polypeptides in \textit{C. reinhardtii} which are immunologically related to either \textit{C. reinhardtii} periplasmic CA or spinach chloroplast CA in wild-type cells. These immunoreactive polypeptides are deficient in a mutant of \textit{C. reinhardtii} which is apparently defective in the induction of the C\textsubscript{i} concentrating system (18).

MATERIALS AND METHODS

Algae Growth

\textit{Chlamydomonas reinhardtii} strain cw15 was obtained from the R. C. Starr algal collection at the University of Texas-Austin, and strain 137 \textit{mt}\textsuperscript{+} has been maintained in the laboratory of R. K. Togasaki. Cells were grown photoautotrophically in suspension in minimal medium (26) with constant shaking while bubbling with air, and were harvested as previously described (16).

CA Assay

CA activity was determined by measuring the rate of H\textsuperscript{+} produced as a result of CO\textsubscript{2} hydration at 4°C (30). An aliquot of a cell suspension (normally 100 \mu L with about 500 \mu g Chl/ml) was added to 3.0 mL of 22 mm Na-barbital (pH 8.3), with constant stirring. The reaction was initiated by the addition of 1.5 mL ice-cold CO\textsubscript{2}-saturated H\textsubscript{2}O. The time required for the pH to change from 8.0 to 7.0 was measured, and the activity was calculated from the equation, \( v = (v_0 - v_t)/t \), where \( t \) is the time required for the pH change when a sample was present, and \( v_0 \) is the time required for the pH change when 100 \mu L of the sample buffer was substituted for the algal sample. A linear relationship between the amount of CA and the measured activity was observed in a range of 0 to 10 units/mL in the assay mixture.

CA activity was measured with harvested cells, resuspended in the 22 mm Na-barbital (pH 8.3) buffer, either before or after lysis by three passages of 5 min each through a Yeda press with 1500 psi. Activity was measured within 20 min after cell lysis.

When the effect of the inhibitors, acetazolamide (Sigma) and ethoxzolamide (a gift from Thomas Maren), on CA activity was examined, small volumes of the inhibitor (20 \mu L or less, dissolved in DMSO) were added to the assay buffer prior to addition of the sample, to provide the appropriate final concentration. At the concentrations of inhibitor utilized, they had no effect on the nonenzymatic rate of CO\textsubscript{2} hydration (\( v_0 \)), and the addition of 20 \mu L of DMSO alone to the assay had no effect on the CA-catalyzed rate.

Isolation of Carbonic Anhydrase

Periplasmic CA from air-grown \textit{C. reinhardtii} (strain 137) was isolated by affinity chromatography with \( \beta \)-methylamino-benzene-sulfonamide-substituted Sepharose 4B, as described by Yang et al. (34). CA was isolated from spinach leaves by the procedure of Kandel et al. (9).

Isolation of Chloroplasts from cw15 Cells

Intact chloroplasts were isolated from gently lysed cw15 cells by Percoll gradient centrifugation as described (3). Stromal fractions were stored frozen until utilized for SDS-PAGE and immunoblotting.

Preparation of Antisera

To assure that trace contaminants were not present in the CA preparations, prior to immunization, the purified CA was subjected to SDS-PAGE, and the band corresponding to CA (26 kD for the spinach enzyme and 36–37 kD for \textit{C. reinhardtii} periplasmic CA) was cut out of the gel. Following collection of premimmune serum, female New Zealand White rabbits were immunized with 100 \mu g of the electrophoretically purified CA in Freund's complete adjuvant (Sigma). For several months, at 2 to 3 week intervals following the initial injection, 50 \mu g of CA was injected in Freund's incomplete adjuvant. Immune sera collected 8 to 10 d after the subsequent injections were pooled and stored at −80°C until use.

Immunoblotting

Samples to be immunoblotted were separated by SDS-PAGE on 12% polyacrylamide gels (0.8% bis), using the electrophoresis buffer system of Laemmlii (12). Samples of algal or stromal extracts corresponding to 80 \mu g Chl were loaded on each lane of the gel. Following electrophoresis, proteins were transferred from the gels to nitrocellulose (Bio-Rad) with a transfer buffer of 20% methanol, 20 mm Tris-glycine, pH 7.5. Dot blots were prepared by spotting 1 \mu L samples on nitrocellulose. Immunoblots were probed with antiserum (diluted 1/500) and detected with horseradish peroxidase conjugated goat-anti rabbit IgG, using the reagents and procedures suggested by Bio-Rad Laboratories (4). The mol wt of electrophoretically separated immunoreactive protein bands were estimated relative to the migration of pre-stained high mol wt standards (Bethesda Research Laboratories) run in parallel.
RESULTS

CA Catalytic Activity

The activity of CA was measured in the cell wall-less mutant of *C. reinhardtii* (cw15), before and after cellular lysis to measure, by difference, the activity of intracellular CA. It has been previously demonstrated that cw15 cells release the periplasmic form of the enzyme into the medium (5, 10). After harvesting and extensively washing of cw15 cells with minimal medium to remove the periplasmic form of the CA which might have remained associated with the cells, a small amount of CA activity could still be detected with the intact cells (Table I). The residual activity remained despite additional washings, and was verified to be extracellular because the activity was completely inhibited by a plasmalemma-impermeant, 9 kD, dextran-bound sulfonamide (17).

After lysis of the washed cw15 cells in a Yeda press, significantly higher levels of CA activity were observed than with intact cells. This increased activity is attributed to one or more intracellular forms of CA. The levels of activity detected with both intact and lysed cells were low, and approached the limit of detection by the CA assay. However, the reproducibility and the sensitivity of this low activity to heat denaturation (not shown) and sulfonamide inhibition (see next section) are consistent with the supposition that the apparent catalytic activity was due to CA.

Sulfonamide Inhibition of *Chlamydomonas* CA

The effect of the sulfonamides, acetazolamide and ethoxyzolamide, on CA activity was examined for both the intracellular CA activity and the periplasmic form of the enzyme. In Figure 1 is shown the effect of varying concentrations of these inhibitors on the activity of the intracellular CA in the lysed cw15 cells, and the periplasmic (extracellular) CA of intact strain 137 cells. The measured $I_{50}$ values for the inhibition of cellular CA, and for purified periplasmic CA and spinach CA, are also listed in Table II. The intracellular CA in cw15 cells was more than three orders-of-magnitude less sensitive to inhibition by both acetazolamide and ethoxyzolamide than was the periplasmic enzyme in intact strain 137 cells, or the isolated periplasmic enzyme. These results, which have been presented at a symposium on C3 transport (6), indicate that one or more forms of CA exist intracellularly which differ from the periplasmic enzyme. At $10^{-6}$ M acetazolamide or ethoxyzolamide, the periplasmic CA was completely inhibited, and the activity in the lysed cw15 cells was also 20 to 30% inhibited, even though this concentration was much less than the $I_{50}$ for the inhibition of the predominant intracellular form of the enzyme (Fig. 1). This 20 to 30% inhibition of the activity of the lysed cells at low concentrations of sulfonamides was likely due to the inhibition of periplasmic CA that was not removed by extensive washing of the cw15 cells, and was consistent with values calculated from the data in Table I, which indicate that 27% (8/30 × 100%) of the observed activity in the lysed cw15 cells could be attributed to the periplasmic form of the enzyme.

The $I_{50}$ value of the periplasmic CA in 137 cells was not significantly affected by the lysis of the cells (Table II). This discounted the possibility that the lysis of the cw15 cells caused the release of some factor which diminished the affinity for sulfonamide binding to the enzyme and the resultant weak inhibition.

Immunological Identification of CA

In higher plants, CA is reported to be localized predominantly in the chloroplast (7, 29) and is weakly inhibited by acetazolamide and ethoxyzolamide (7, 9, 23). Similarly, the intracellular CA activity in *C. reinhardtii* was relatively insensitive to inhibition by sulfonamides as compared to the peri-

![Figure 1](image-url). Inhibition of CA activity in intact strain 137 cells (·) and in lysed cw15 cells (○) of *reinhardtii*.

**Table II. Sulfonamide Inhibition of Intracellular and Periplasmic (Extracellular) CA in *C. reinhardtii* and Spinach Carbonic Anhydrase**

<table>
<thead>
<tr>
<th>CA</th>
<th>Inhibitor</th>
<th>$I_{50}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular (lysed cw15 cells)</td>
<td>Acetazolamide</td>
<td>$3 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>Ethoxyzolamide</td>
<td>$2 \times 10^{-5}$</td>
</tr>
<tr>
<td>Periplasmic (intact 137 cells)</td>
<td>Acetazolamide</td>
<td>$8 \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>Ethoxyzolamide</td>
<td>$6 \times 10^{-9}$</td>
</tr>
<tr>
<td>Periplasmic (lysed 137 cells)</td>
<td>Acetazolamide</td>
<td>$9 \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>Ethoxyzolamide</td>
<td>$8 \times 10^{-9}$</td>
</tr>
<tr>
<td>Periplasmic (purified)</td>
<td>Acetazolamide</td>
<td>$8 \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>Ethoxyzolamide</td>
<td>$5 \times 10^{-9}$</td>
</tr>
<tr>
<td>Spinach CA (purified)</td>
<td>Acetazolamide</td>
<td>$3 \times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td>Ethoxyzolamide</td>
<td>$5 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

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**Table I. Activity of CA in Intact and Lysed *C. reinhardtii* Cells**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>CA Activity</th>
<th>CA Activity</th>
<th>CA Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Washed intact cells</td>
<td>Lysed cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>units/mg Chl</td>
<td></td>
</tr>
<tr>
<td>Wall-less, cw15</td>
<td>8 ± 6 (4)</td>
<td>30 ± 8 (6)</td>
<td></td>
</tr>
<tr>
<td>137</td>
<td>610 ± 180 (7)</td>
<td>630 ± 220 (4)</td>
<td></td>
</tr>
</tbody>
</table>
plasmic C. reinhardtii enzyme (Table II). Thus, the intracellular CA in C. reinhardtii with a low affinity for sulfonamides might be similar to the CA which exists within the chloroplasts of higher plants. To further test this hypothesis, antisera were prepared against both purified spinach leaf CA and C. reinhardtii periplasmic CA, and were used to identify proteins in C. reinhardtii which were immunologically related to these enzymes.

Dot blots of both isolated spinach CA and C. reinhardtii periplasmic CA, probed with either anti-spinach CA antiserum or anti-C. reinhardtii periplasmic CA antiserum, indicated that the anti-spinach CA antiserum was specific for spinach CA, and did not bind the C. reinhardtii periplasmic CA under the conditions utilized in these experiments (Fig. 2). However, weak cross-reactivity could be observed with higher amounts of the antigen or with more concentrated antibody solutions (data not shown). The anti-C. reinhardtii periplasmic CA antiserum shows less species specificity as it bound spinach CA in addition to the C. reinhardtii periplasmic CA. A similar species specificity has been observed with a monoclonal antibody against the C. reinhardtii periplasmic CA (11). These results differ from those of Yang et al. (34) and our initial report (6) which indicated no species cross-reactivity between the anti-C. reinhardtii periplasmic CA antiserum, and spinach CA. The difference may be due in part to different antisera preparations with different antibody titers.

The antisera were used to probe crude extracts of C. reinhardtii strain 137 (wild-type) or chloroplasts isolated from C. reinhardtii cw15 cells following SDS-PAGE and transfer to nitrocellulose (Fig. 3). In the fractions probed with the anti-C. reinhardtii CA antiserum, the predominant polypeptides of 36 to 37 kD that are characteristic of the periplasmic CA, were evident in whole cell lysates from strain 137 cells, but as expected, were absent from the isolated chloroplasts from cw15 cells. A predominant immunoreactive polypeptide was observed at 45 kD in whole cell extracts or in isolated chloroplast stromal fractions, when probed with either antiserum, indicating that the 45 kD protein may be an intracellular form of CA localized in the chloroplast stroma. Another predominant immunoreactive polypeptide was observed at 110 kD, and was particularly evident in whole cells probed with the anti-spinach CA. Because this polypeptide was present in only low levels in the chloroplast fraction and was not a component of the periplasmic form of the enzyme, it is tentatively considered to have a predominantly cytosolic localization since no evidence has been obtained for a 110 kD periplasmic form of CA (5). A number of other weakly immunoreactive bands were also observed on these immunoblots. A considerable number of minor immunoreactive polypeptides were also observed when extracts were probed with a monoclonal antibody prepared against periplasmic CA (M Kitayama, RK Togasaki, unpublished data). No significant immunoreactive polypeptides were observed when extracts were probed with non-immune serum (Fig. 3).

**DISCUSSION**

Several lines of evidence have been presented for the existence of intracellular CA in *Chlamydomonas reinhardtii*. There is a statistically significant level of measurable CA activity which is released only upon lysis of the cell (Table I). The total intracellular activity, as the difference between the CA activities in the lysed and intact cw15 cells, is 22 units/mg Chl. Assuming that the C. reinhardtii chloroplast occupies about 50% of the cell volume of about 150 μL/mg Chl (2), then the level of CA within the chloroplast stroma of about 290 units/ml could accelerate the rate of CO₂/HCO₃⁻ interconversion within the chloroplast by 30 times the rate which could occur nonenzymatically at the assay temperature of 4°C, and even greater catalytic rates would be expected at higher temperatures. Thus, the intracellular CA activity would seem to be sufficient to be physiologically significant in these algae.

Measurements of the difference in CA activity between lysed and intact C. reinhardtii cells for intracellular CA activity have also been reported by others (25, 28). The value reported here (22 units/mg Chl) is in the range of the intracellular levels of 52 units/mg Chl calculated from the data of Spalding et al. (25) for cw15 cells, but less than the values of 142 to 184 units/mg Chl measured by Tsuzuki et al. (28) for other strains of C. reinhardtii. The detection of CA activity with intact cells is not in itself sufficient evidence of extracellular CA activity, because the substrate, CO₂, is presumed to be freely permeant to the plasma membrane. The presumption that the activity observed with intact cells is only attrib-
utable to the extracellular enzyme is based on the assumption that the reaction product measured in the assay, \( \text{H}^+ \), is impermeant to the plasma membrane under the assay conditions utilized. The low activity observed with well-washed intact cw15 cells is apparently due to residual amounts of the extracellular enzyme, because the activity was inhibited by a membrane-impermeant high molecular weight (9 kD) sulfonamide, and because the activity, like the periplasmic CA, was highly sensitive to inhibition by acetazolamide and ethoxzolamide.

The differential inhibition of the intracellular CA and periplasmic CA to inhibition by the sulfonamides, acetazolamide and ethoxyzolamide, was consistent with the proposal that the two forms of the enzyme differ. This eliminates the possibility that the intracellular activity measured after lysis of the cw15 cells was due to the activation of some contaminating periplasmic enzyme. Similarly, Yagawa et al. (33) have identified high levels of an intracellular CA in a unicellular red alga which lacks a periplasmic CA, *Porphyridium cruentum* R-1. The intracellular enzyme from *P. cruentum* was also weakly inhibited by acetazolamide and ethoxyzolamide, and it was reported to be localized within the chloroplast (33).

When we previously measured the concentration dependence for the sulfonamide inhibition of photosynthetic \( \text{O}_2 \) evolution (17) in *C. reinhardtii* at limiting \( \text{Ci} \) concentrations, the permeant CA inhibitor, ethoxyzolamide, inhibited the efficient utilization of \( \text{Ci} \) in a biphasic manner. At low concentrations of ethoxyzolamide (less than \( 10^{-6} \) M), a similar pattern of inhibition was observed with an apparently impermeant inhibitor, acetazolamide. This result was attributed to the inhibition of the periplasmic CA, which has a high affinity for both inhibitors (Table II). However, at higher concentrations of these two inhibitors, further inhibition of \( \text{O}_2 \) evolution at limiting concentrations of \( \text{Ci} \) was observed only with the permeant inhibitor, ethoxyzolamide. The \( \text{IC}_{50} \) for the second phase of inhibition by ethoxyzolamide was at about 2 to \( 5 \times 10^{-5} \) M (17). Since ethoxyzolamide is thought to be impermeant to biological membranes (15), the concentration within the chloroplast may be similar to that outside of the cell. Thus, those results are consistent with the \( \text{IC}_{50} \) value of \( 2 \times 10^{-5} \) M for the intracellular form of CA when measured in lysed cw15 cells (Table II), which may be due to a physiologically important form of CA within the chloroplast.

In this study we have identified polypeptides in cells and in isolated chloroplasts from *C. reinhardtii*, which were immunoreactive with antisera prepared against spinach and *C. reinhardtii* CA. The cross-reactivity between the spinach chloroplast CA and the anti-*C. reinhardtii* CA antisera indicate structural similarities between CA in these species, yet the anti-spinach CA antibody reacted only very weakly with *C. reinhardtii* periplasmic CA. Several polypeptides were observed in cellular extracts which were immunoreactive with both the anti-spinach CA antiserum and the anti-*C. reinhardtii* periplasmic CA antiserum, notably a 45 kD polypeptide which was apparently localized in the chloroplast stroma, and a 110 kD polypeptide which may be in the cytoplasm. These particular polypeptides bind both anti-spinach and anti-*C. reinhardtii* CA antisera, consistent with their proposed identity as CA or structurally related proteins. Anti-*C. reinhardtii* periplasmic CA IgG or anti-spinach CA IgG did not inhibit the activity of the intracellular CA activity when measured 30 min after the addition of the antibodies to the extract containing the enzyme. However, because of the instability of the intracellular CA activity, we have been unable to test the inhibition or immunoprecipitation of the intracellular CA activity after the longer periods of time which may be necessary for the antibody-antigen complex to form. The low activity of intracellular CA has made the measurement of CA catalytic activity in isolated chloroplast preparations difficult due both to the insensitivity of available methods for the measurement of CA activity and to the limited quantities of isolated chloroplast preparations that can be prepared. Thus, positive identification of the intracellular CA catalytic activity as chloroplastic in location and its identity with one or more of the immunoreactive polypeptides observed in the immunoblots has yet to be established. However, the deficiency of the 45 kD and 110 kD immunoreactive polypeptides in the *cia-5* mutant *C. reinhardtii* which is apparently defective in the induction of the C, concentrating system and both intracellular and periplasmic forms of CA (18), supports the contention that these polypeptides are CA and/or components of the C, concentrating system.

Other evidence supports the involvement of polypeptides near 45 kD with the C, concentrating system of *C. reinhardtii*. Polypeptides of 44 and 46 kD that were immunoreactive with monoclonal antibodies prepared against periplasmic CA were induced when cells were transferred from high CO\(_2\) to air levels of CO\(_2\) (M Kitayama, RK Togasaki, unpublished). Also, the induction of the synthesis of 44 and 46 kD polypeptides is observed by *in vivo* labeling of proteins with \( \text{35}^{\text{S}}\text{SO}_4^{2-} \), upon induction of the C, concentrating system (13). The relationship between the polypeptides remains to be established.

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**LITERATURE CITED**


