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Chemical Modification of the Functional Arginine Residues of Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*†

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ABSTRACT: Carbon monoxide dehydrogenase (CODH) is the key enzyme of autotrophic growth with CO or CO_2 and H_2 by the acetyl-CoA pathway. The enzyme from *Clostridium thermoaceticum* catalyzes the formation of acetyl-CoA from the methyl, carbonyl, and CoA groups and has separate binding sites for these moieties. In this study, we have determined the role of arginine residues in binding of CoA by CODH. Phenylglyoxal, an arginine-specific reagent, inactivated CODH, and CoA afforded about 80–85% protection against this inactivation. The other ligands, such as the carbonyl and the methyl groups, gave no protection. By circular dichroism, it was shown that the loss of activity is not due to extensive structural changes in CODH. Earlier, we showed that tryptophan residues are located at the CoA binding site of CODH [Shanmugasundaram, T., Kumar, G. K., & Wood, H. G. (1988) *Biochemistry 27*, 6499–6503]. A comparison of the fluorescence spectra of the native and phenylglyoxal-modified enzymes indicates that the reactive arginine residues appear to be located close to fluorescing tryptophans. Fluorescence spectral studies with CoA analogues or its components showed that CoA interacts with the tryptophan(s) of CODH through its adenine moiety. In addition, evidence is presented that the arginines interact with the pyrophosphate moiety of CoA.

Wood and his collaborators discovered a pathway of acetyl coenzyme A (acetyl-CoA)[1] synthesis in *Clostridium thermoaceticum* which differs from all previously described pathways for autotrophic growth such as the Calvin cycle or the reductive citric acid cycle. Most of the enzymes involved in various steps of the pathway have been purified [see Wood et al. (1986a–c) and Ljungdahl (1986) for reviews]. Carbon monoxide de-

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[1] Abbreviations: acetyl-CoA, acetyl coenzyme A; CODH, carbon monoxide dehydrogenase; DTT, dithiothreitol; CoA, coenzyme A; CD, circular dichroism; DNPS-Cl, 2,4-dinitrophenylsulfenyl chloride.

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hydrogenase (CODH), the key enzyme in this pathway, consists of two subunits with $\alpha_3\beta_3$ structure, and it has separate binding sites for CO, CoA, and the methyl groups (Ragsdale & Wood, 1985). It has been shown that a Ni–Fe center is involved during the interaction of CO with CODH (Ragsdale et al., 1985). Also, Pezacka and Wood (1988) have shown that the methyl group is bound to a cysteine residue on the β subunit of CODH. Further, they have reported that methyl iodide can serve as a methyl donor for the synthesis of acetyl-CoA. Recently, it has been shown that tryptophan residues are located at or near the CoA binding site of CODH (Shanmugasundaram et al., 1988a). Earlier, Ragsdale and Wood (1985) showed in preliminary studies that phenylglyoxal and butanedione inhibit the exchange activity catalyzed by CODH. In order to identify the residues involved in CoA binding and catalysis, we have studied the effect of phenylglyoxal modification on the activity of CODH both in the presence and in the absence of CoA.

Materials and Methods

Materials

Phenylglyoxal, pantothenic acid, adenine, D-ribose, and methyl iodide were obtained from Aldrich. CoA, 3′-dephospho-CoA, and sodium pyrophosphate were from Sigma. Cysteamine was from Fluka.

Methods

CODH was purified from *C. thermoaceticum* under anaerobic conditions as described by Ragsdale and Wood (1985). The acetyl-CoA synthesis activity was monitored as described earlier (Hu et al., 1984; Shanmugasundaram et al., 1988b). The total number of reactive arginine residues in CODH was determined by the method of Yamasaki et al. (1981).

Phenylglyoxal Modification of CODH. Phenylglyoxal modification of CODH was carried out as described in the legend of Figure 1.

Circular Dichroism. The circular dichroism spectra were recorded with a Jasco Model J-40A spectropolarimeter (Jasco, Inc., Easton, MD) calibrated with *d*-10-camphorsulfonic acid (Cassim & Young, 1969). Samples were placed in an anaerobic cylindrical quartz cell with a path length of 1 mm. The mean residue ellipticity values, $[\theta]$, were calculated by using

$$[\theta] = HS(\mathrm{MRW})/Cl \qquad (1)$$

where H is the height of the peak, S is the sensitivity, MRW is the mean residue weight ($\sim$115), C is the concentration of the protein in grams per liter, and l is the path length of the cell. The protein content was estimated by the Rose Bengal assay (Elliot & Brewer, 1978).

Fluorescence Measurements. Fluorescence emission spectra of CODH were recorded by using an excitation wavelength of 295 nm with a Shimadzu RF-540 spectrofluorometer by scanning from 300 to 400 nm. Fluorescence measurements were made in the range where fluorescence emission was linear with protein concentration. Suitable aliquots of the stock solution of CODH were transferred anaerobically by using a gas-tight syringe into an anaerobic cell containing appropriate volumes of buffer. A degassed 20 mM sodium phosphate buffer, pH 8.2, containing 4 mM DTT was used for dilution. The fluorescence was corrected for dilution and inner filter effects (Hélène et al., 1969). The fluorescence spectra of CODH were also recorded in the absence or presence of CoA analogues or its components such as cysteamine, pyrophosphate, 3′-dephospho-CoA, pantothenic acid, adenine, and ribose.

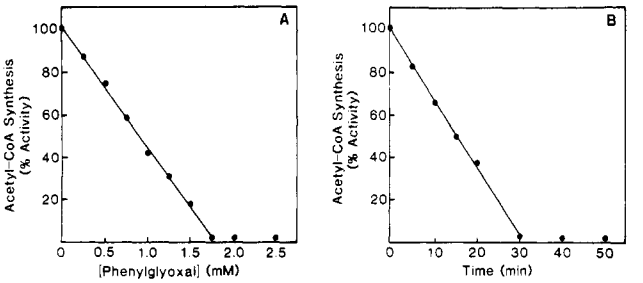

FIGURE 1: (A) Effect of concentrations of phenylglyoxal on the acetyl-CoA synthesis catalyzed by CODH. CODH (2 mg/mL) was incubated with varied concentrations of phenylglyoxal in 20 mM sodium phosphate buffer, pH 8.2, containing 4 mM DTT at 25 °C. After 30-min incubation under anaerobic conditions in tightly sealed tubes, the reaction mixture was passed through a Sephadex G-25 column to remove the excess reagent. The acetyl-CoA synthesis was carried out in a total volume of 0.5 mL containing either native or modified CODH (0.18 mg), ferredoxin (0.02 mg), corrinoid protein (0.1 mg), methyltransferase (0.1 mg), CoA (0.8 μmol), and methyl tetrahydrofolate (2.0 μmol; 709 cpm/nmol) in 100 mM potassium phosphate buffer, pH 6.0, containing 5 mM DTT under a CO atmosphere. (B) Time course of inactivation of CODH by phenylglyoxal. CODH (4.6 μM) was incubated with 1.75 mM phenylglyoxal in 20 mM sodium phosphate buffer, pH 8.2, containing 4 mM DTT at 25 °C. At times indicated, aliquots were removed for measurement of the residual enzyme activity as described above.

Table I: Modification of Arginine Residues of CODH in the Presence and Absence of Substrates[a]

sample	no. of arginines modified	activity remaining (%)
CODH	6	2
CODH + 10 μM CoA	4	85
CODH + CO + CH_3I[b]	6	2
CODH[c]	67.5	ND

[a] CODH (2 μM) was incubated with 1.75 mM *p*-nitrophenylglyoxal in 20 mM sodium phosphate buffer, pH 8.2, containing 4 mM DTT at 25 °C. After 30-min incubation, aliquots were taken for the estimation of arginine content and also for residual activity determination. The number of arginines modified were calculated per 440 000 g of enzyme. [b] The concentration of methyl iodide was 2 μM, and the medium was saturated with CO. [c] Determined by using the tryptic digest of CODH.

Results

Inactivation of CODH by Phenylglyoxal. Phenylglyoxal and *p*-nitrophenylglyoxal are known to modify arginine residues in proteins (Means & Feeney, 1973; Yamasaki et al., 1981). Preincubation of CODH with increasing concentrations of phenylglyoxal at room temperature (for 30 min) resulted in an irreversible loss of acetyl-CoA synthesis activity in a concentration-dependent manner (Figure 1A). There is a linear loss of activity up to 1.75 mM phenylglyoxal. At a concentration of 1.75 mM, *p*-nitrophenylglyoxal also produced about 98% inactivation (Table I), and the resultant chromogenic *p*-nitrophenylglyoxal product was used for the colorimetric determinations of arginine residues. By this analysis, it was estimated that six arginine residues were modified per mole of CODH (Table I). A typical time course of inactivation of CODH using a fixed concentration of phenylglyoxal is shown in Figure 1B. The synthesis of acetyl-CoA was abolished linearly with time. Thus, at 30-min incubation time, 85–95% inactivation was observed with phenylglyoxal while under similar conditions of incubation of control CODH without phenylglyoxal loss of enzyme activity was not observed.

Treatment of CODH with Phenylglyoxal or p-Nitrophenylglyoxal in the Presence of Substrates. The effect of various substrates on the inactivation of CODH was examined

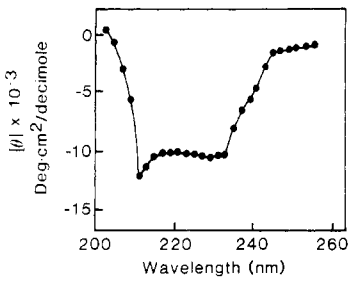

FIGURE 2: Circular dichroism spectra of native CODH and phenylglyoxal (1.75 mM) modified CODH. Dilute solutions of CODH, 0.46 μM, were used in 20 mM sodium phosphate buffer, pH 8.2, containing 4 mM DTT. The curves are superimposed on each other.

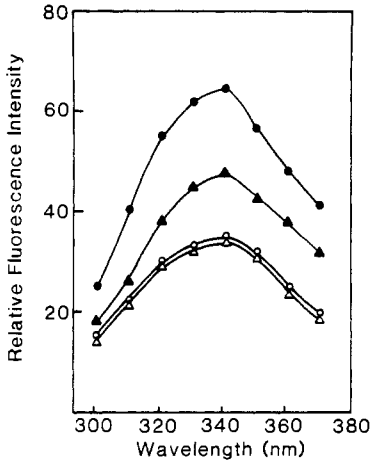

FIGURE 3: Fluorescence spectra of the native (●) CODH and phenylglyoxal-modified CODH at concentrations of phenylglyoxal of 1.0 mM (▲), 1.75 mM (○), and 2.5 mM (△) modified CODH. Dilute solutions of CODH, 0.23 μM, were used in 20 mM sodium phosphate buffer, pH 8.2, containing 4 mM DTT. The fluorescence spectrum of CODH treated with phenylglyoxal (1.75 mM) was not altered by 10 μM CoA.

to determine the target of inactivation. CoA (at a concentration as low as 10 μM) protected (80–85%) the enzyme against phenylglyoxal inactivation, but the other substrates, viz., CO and CH_3I, failed to protect the enzyme. In the presence of 10 μM CoA, about four arginine residues were modified (Table I). Thus, CoA provided protection against modification of two arginine residues. The above results indicate that certain reactive arginine residues are located at or near the CoA binding site of CODH.

Inactivation Caused by Phenylglyoxal Is Not Due to Extensive Structural Change in the Enzyme. The CD spectra (Figure 2) were identical with the untreated and phenylglyoxal-treated CODH, and the elution behavior of the native and phenylglyoxal-modified enzymes was the same on a column of Bio-Gel A 0.5M (data not shown). These results provide evidence that the inactivation caused by phenylglyoxal is not due to a gross change either in the secondary or in the quaternary structure of the enzyme.

Fluorescence Spectra of the Native and Phenylglyoxal-Modified CODH. The fluorescence spectra of the native and phenylglyoxal-modified enzymes are shown in Figure 3. As a result of phenylglyoxal modification, the fluorescence of the enzyme was quenched by about 50% without any significant change in the emission maximum. Thus, the modification of the arginines by phenylglyoxal appears to perturb the microenvironment of certain tryptophans. Earlier, it has been shown that CoA produced quenching (~50%) of the fluorescence of CODH (Shanmugasundaram et al., 1988b). In the present study, it has been observed that in the presence of CoA,

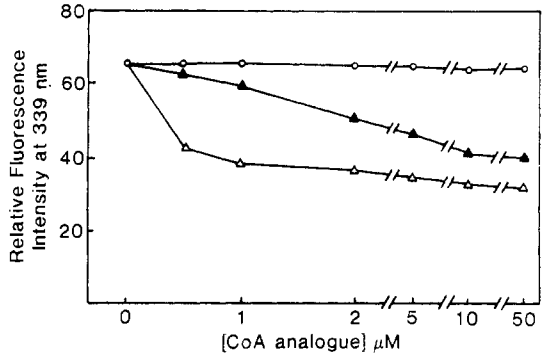

FIGURE 4: Effect of the components and analogue of CoA on the fluorescence emission maximum of CODH: adenine (▲), 3′-dephospho-CoA (△), pantothenic acid or cysteamine or ribose or inorganic pyrophosphate (○). CODH (0.23 μM) in 20 mM sodium phosphate buffer, pH 8.2, containing 4 mM DTT was used in these experiments. Excitation wavelength was 295 nm.

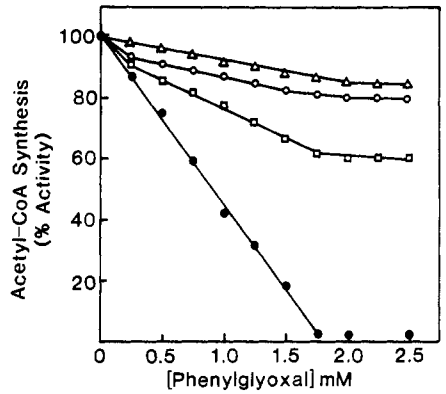

FIGURE 5: Effect of CoA, its components, and 3′-dephospho-CoA on the phenylglyoxal-mediated inactivation of CODH. CODH (4.6 μM) was incubated with various concentrations of phenylglyoxal in the presence (△) and absence (●) of 10 μM each of CoA and 3′-dephospho-CoA (○) and 50 μM inorganic pyrophosphate (□). After 30-min incubation, aliquots were taken for the measurement of the residual enzyme activity as described in the legend of Figure 1. The same results were obtained when the reaction was carried out at pH 8.2 with Tris-HCl buffer.

phenylglyoxal does not produce any changes in the fluorescence spectrum of CODH. It, therefore, appears that the phenylglyoxal-reactive arginine residues are located close to the tryptophan residues at the CoA binding site.

Mode of CoA Binding with CODH. On the basis of the results presented here and earlier (Shanmugasundaram et al., 1988b), it appears that certain arginine and tryptophan residues are involved in CoA binding with CODH. In order to determine the mode of CoA binding with these residues in CODH, fluorescence and phenylglyoxal modification studies were carried out with the components or analogues of CoA such as 3′-dephospho-CoA, cysteamine, pantothenic acid, adenine, ribose, and inorganic pyrophosphate. Of these CoA moieties, only adenine and 3′-dephospho-CoA interact with the tryptophan residue(s) of CODH as evidenced by the quenching of the tryptophan fluorescence of the enzyme (Figure 4). The observed quenching with adenine suggests that when CoA binds, the adenine moiety of CoA interacts with the tryptophan(s) of CODH. However, with phenylglyoxal modification studies with CoA analogues or its components, it was observed that only inorganic pyrophosphate and 3′-dephospho-CoA protected the enzyme against inactivation (Figure 5). Thus, it appears that the pyrophosphate bridge of CoA may be bound to the arginine residue(s) of CODH.

Discussion

It is necessary to satisfy at least the following criteria to implicate a specific amino acid residue in the catalytic function of enzyme mechanism: (i) modification of the particular residue should result in loss of enzyme activity, and (ii) masking the active center with a substrate or substrate analogue should significantly reduce the inhibition. Arginines at the active site of CODH meet these criteria in the following manner.

CODH is inactivated by the arginine-specific reagent phenylglyoxal. Considerable protection (80–85%) is provided against this inactivation by CoA but not by the methyl and carbonyl groups. These results suggest that arginine residues are located at or near the CoA binding site of CODH.

Since all arginine residues are not necessarily reactive in the native state (Yamasaki et al., 1981), the total number of arginine residues were estimated in the tryptic digest of CODH. About 68 arginine residues are present in CODH (Table I), which has a molecular weight of 440 000 and a subunit stoichiometry of $\alpha_3\beta_3$ (Ragsdale et al., 1983). In the native state, about six arginine residues were modified. In the presence of CoA, only four arginine residues were modified by phenylglyoxal. This result supports the view that at least two of the exposed, reactive arginine residues are essential for the catalytic activity of CODH.

In addition to arginine residues, phenylglyoxal can also react with the sulfhydryl and ϵ-amino groups in proteins (Means & Feeney, 1973). A comparison of the amino acid composition of the native and modified CODH's (data not shown) indicated that only arginine residues were modified by phenylglyoxal. In addition, the phenylglyoxal-mediated inactivation apparently is not due to secondary or quaternary structural alterations of CODH as evidenced by CD and exclusion chromatographic studies.

Previously, we have shown that certain tryptophans are located at or near the CoA binding site of CODH and the binding of CoA caused significant quenching of the fluorescence of CODH (Shanmugasundaram et al., 1988a). In the present study, we have observed that phenylglyoxal treatment produced quenching of the fluoresence of CODH. Also, it has been observed that CoA did not produce further quenching of the fluorescence of the phenylglyoxal-treated enzyme. These results suggest that apart from the essential tryptophan(s), arginine residues are also involved in CoA binding with CODH.

Fluorescence studies with CoA analogues or its components suggested that the adenine moiety of CoA interacts with the tryptophan(s). This is evidenced by quenching of the fluorescence of CODH by adenine. On the other hand, the phenylglyoxal modification studies indicate that the inorganic pyrophosphate bridge of CoA binds to the arginine residue(s) of CODH as evidenced by the protection of CODH against phenylglyoxal modification by 3′-dephospho-CoA and inorganic pyrophosphate. Remington et al. (1982), using crystallographic studies, have shown that the pyrophosphate and the 3′-phosphate moieties of CoA interact with the arginine residues of citrate synthetase. However, we have observed that the 3′-phosphate group is not essential for CoA binding with CODH and the 3′-dephospho-CoA is able to replace CoA in the net catalytic reaction (Shanmugasundaram and Wood, unpublished results). A similar result was observed by Cha et al. (1964) for succinyl-CoA synthetase. Arginine residues are proposed to be involved in the active site of CODH from *Methanobacterium barkerri* (Grahame & Stadtman, 1987). Detailed studies by Lange and his co-workers have shown that the pyrophosphate bridge of coenzymes can be bound to the arginine residue via the guanidino groups (Lange et al., 1974). Chandrasekar and Plapp (1988) have also shown by X-ray crystallography that the pyrophosphate bridge of the coenzyme NAD binds to the guanidinium groups of arginine residues of horse liver alcohol dehydrogenase. On the basis of these studies, it appears in general that CoA binds to the arginine residues of CODH in a manner similar to various coenzyme-dependent enzymes. Recently, Raybuck et al. (1988), by kinetic studies, have shown that CoA also interacts with the metal centers of CODH through the sulfur atom.

Chemical modification and fluorescence studies thus have shown that tryptophan and arginine residues are involved in CoA binding with CODH. It will be of great interest to isolate and sequence the CoA binding site peptide(s) by labeling one of these residues by using fluorescent or radioactive probes. In this context, 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl) appears to be a promising fluorescent probe (Scoffone et al., 1969), which has been successfully used in the isolation of an essential tryptophan-containing peptide of the pyruvate binding site of transcarboxylase (Kumar et al., 1988). Currently, studies involving the labeling of the tryptophan residues of CODH with DNPS-Cl in the presence and absence of CoA are under way with the goal of determining the amino acid sequence(s) at the CoA binding site.

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Activation of $O_2^{\cdot -}$ Generating Oxidase of Bovine Neutrophils in a Cell-Free System. Interaction of a Cytosolic Factor with the Plasma Membrane and Control by G Nucleotides†

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ABSTRACT: Activation of the $O_2^{\cdot -}$-generating oxidase of bovine neutrophils was studied in a cell-free system, consisting of a particulate fraction enriched in plasma membrane, cytosol, arachidonic acid, and the nonhydrolyzable nucleotide GTP-γ-S. Activation of the membrane-bound oxidase was accompanied by the disappearance of the activating factor from the cytosol. Above a cytosol to membrane ratio of 25, the excess of added cytosolic factor remained in active state in the soluble fraction. The process could be partially reversed by serum albumin. Disappearance of the cytosolic factor was promoted by unsaturated long-chain fatty acids, but not by saturated ones, and occurred not only in the presence of GTP-γ-S but also in the presence of GDP-β-S or in the absence of Mg ions, although in the latter cases activation of $O_2^{\cdot -}$ production was seriously impaired. This suggests that the disappearance of the activating factor from the cytosol and the triggering effect of GTP-γ-S are related, but distinct, events in the oxidase activation process. The disappearance of the activating factor from cytosol can be explained by translocation of the cytosolic factor to the membrane fraction. Yet under some conditions, including the presence of GDP-β-S or EDTA, inactivation was prevailing and could be an alternative explanation for the results. Specific binding of radiolabeled GTP-γ-S could be demonstrated both in the membrane and in the cytosolic fractions. Although a substantial amount of GTP-γ-S was able to bind to the membrane proteins, its effect on oxidase activation was moderate compared to that of GTP-γ-S bound to cytosolic proteins. Oxidase activation was correlated with the binding of GTP-γ-S to cytosolic proteins in a range of concentrations from approximately 0.3 to 2.5 μM GTP-γ-S, with a GTP-γ-S concentration of $\simeq 1$ μM corresponding to the half-maximal oxidase activation. A GTP-binding protein of $M_r = 23\,000$ was detected in cytosol; its function in oxidase activation remains to be assessed.

The enzyme responsible for the respiratory burst in phagocytosing cells is a NADPH-specific oxidase located in plasma membrane. It catalyzes the one-electron transfer from NADPH to O_2 [for review see Rossi (1986) and Bellavite (1988)]. It is dormant in resting cells and is activated by a number of extracellular stimuli. A breakthrough in the study of the mechanism of oxidase activation was the development of a cell-free system of activation. This technique has been applied to macrophages and neutrophils of various species (Bromberg & Pick, 1984, 1985; McPhail et al., 1985; Curnutte, 1985; Gabig et al., 1987; Ligeti et al., 1988; Tanaka et al., 1988). It consists of incubation of a membrane fraction enriched in plasma membrane with cytosol in the presence of long-chain unsaturated fatty acids (Seifert & Schultz, 1987; Cox et al., 1987; Curnutte, 1985; Gabig et al., 1987; Ligeti et al., 1988) or SDS[1] (Bromberg & Pick, 1985; Pick et al., 1987; Cox et al., 1987; Babior et al., 1988). Both the rate of activation and the maximal oxidase activity achieved were found to depend on the amount of cytosol present during the incubation (Ligeti et al., 1988). This behavior suggested that there is a stoichiometric reaction between membranous and cytosolic components. Furthermore, the observation that the $O_2^{\cdot -}$-producing capacity of the membrane fraction persists after its separation from the cytosol (Gabig et al., 1987; Doussière et al., 1988) implies that the oxidase undergoes a quasi-irreversible modification after the transition from the resting state to the activated state. The possibility of the formation of a complex between cytosolic and membrane proteins was raised (Sha'ag & Pick, 1988; Doussière et al., 1988; Tanaka et al., 1988), but neither the conditions of the translocation of the activating factor from the cytosol to the membrane nor its eventual dissociation from the membrane and reappearance in cytosol were rigorously tested.

Activation in the cell-free system is stimulated by GTP-γ-S and fluoride and inhibited by GDP or GDP-β-S and depletion

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-Mg, PBS supplemented with 2.5 mM $MgCl_2$.

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