Regulation of Porphyrin Biosynthesis

PURIFICATION AND CHARACTERIZATION OF δ-AMINOLEVULINIC ACID SYNTHASE*

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SUMMARY

δ-Aminolevulinic acid synthase has been purified 1300-fold from photosynthetically cultured Rhodopseudomonas spheroides. The enzyme having a specific activity of 130 units per mg shows one major band and two minor bands upon disc electrophoresis. Some properties, including molecular weight, stability, and the inhibition of enzyme activity by hemin are reported. The comparative behavior of crude and purified enzyme toward oxidizing and reducing agents is discussed. Although δ-aminolevulinic acid synthase functions in a regulatory capacity, it apparently lacks some characteristics generally observed with allosteric enzymes.

The first step in the biosynthesis of tetrapyroles involves the condensation of glycine and succinyl-CoA to yield δ-aminolevulinic acid and carbon dioxide. The enzyme that catalyzes this reaction, δ-aminolevulinic acid synthase (succinyl-CoA; glycine succinyltransferase) has been shown in a wide variety of systems (1-4). It is generally accepted that this enzyme plays a major role in regulating the biosynthesis of porphyrins. δ-Aminolevulinic acid synthase is a repressible enzyme, and it is inhibited by hemin in what appears to be the classical feedback manner (5-7). There is evidence that factors other than hemin also affect the activity of δ-aminolevulinic acid synthase, at least under certain circumstances (8-10). To date none of the nonheme factors have been identified, although the evidence indicates that these factors concern the oxidation-reduction state of the cell or enzyme extract (8, 9, 11).

We have for some time been working on the purification of δ-aminolevulinic acid synthase from the photosynthetic bacterium Rhodopseudomonas spheroides in the belief that a full understanding of this enzyme will only be possible when it is available in pure form and in reasonable quantity. We have been hampered in our efforts by the instability of the enzyme as well as by its sometime erratic behavior in crude preparations. Nevertheless, we have now succeeded in purifying δ-aminolevulinic acid synthase 1300-fold with a 10% yield.

EXPERIMENTAL PROCEDURE

Materials—Protamine sulfate, grade II, lot number 79B-1420 and 30B-1450; coenzyme A, grade 1-L; pyridoxal phosphatate; ATP and δ-aminolevulinic acid were purchased from Sigma. Bovine serum albumin and disodium succinate were purchased from Calbiochem. Enzyme grade ammonium sulfate was from Mann. DEAE-cellulose, type Cellex-D and hydroxylapatite, type Bio-Gel HT, were products of Bio-Rad Laboratories, Richmond, California. Ultrafiltration membranes, type UM20EF, were purchased from the Amicon Corporation, Cambridge, Massachusetts.

Calcium phosphate gel for column chromatography was prepared according to the method of Mathews, Brown, and Cohen (12). Succinic thiokinase, free of δ-aminolevulinic acid synthase activity, was prepared according to the procedure of Burnham (13). Rabbit apohemopexin was a generous gift of Dr. Ursula Muller-Eberhard.

Growth of Cells—R. spheroides (NCIB 8253) obtained from Dr. June Lascelles has been maintained in stab culture with periodic transfer. Growth was in Lascelles Medium S (14) in 9-liter bottles on a rotating incubator at 25° and at a light level at which the cultures reached stationary phase in 48 hours (one 40-watt incandescent bulb per bottle at a distance of 20 cm). After 36 hours growth the cells were cooled to 4°, harvested by centrifugation, and resuspended in 100 mM Tris-chloride buffer, pH 7.5, containing 10% glycerol, 1 mM 2-mercaptoethanol, and 0.5 mM EDTA to a concentration of 250 mg of cells, dry weight, per ml. Concentration in dry weight of cells per ml was determined by the absorbance at 660 nm compared to a standard curve. Cells were stored at -15° until used.

Enzyme Assays—The assay procedure used for δ-aminolevulinic acid synthase has been described previously (11). Compounds, such as, potassium ferricyanide or dithiothreitol, which were tested for their effect on δ-aminolevulinic acid synthase activity, were usually added directly to a portion of the enzyme prior to addition of the enzyme to the assay mixture. Control samples were run to which the test compound was added at the termination of incubation and color corrections were applied when necessary.

Definition of Enzyme Activity—One unit of δ-aminolevulinic acid synthase catalyzes the formation of 1 amole of δ-aminolevulinic acid per hour under these assay conditions. Specific activity
is expressed in units per mg of protein. During some characterization studies, preparations containing 60 to 80 units per mg were used while the remainder were performed with preparations containing 130 units per mg. Spot checks were done comparing the results, i.e., $K_v$ values, obtained with both preparations and the results were always in agreement.

**Determination of Protein**—Protein was determined by the method of Lowry et al. (15) with bovine serum albumin as standard. In later stages of the purification, protein was determined spectrophotometrically by the absorbance at 280 nm.

**Electrophoresis**—Polyacrylamide gel electrophoresis was generally done with the discontinuous pH 8.9 system of Davis with the E-C vertical gel electrophoresis apparatus (16). Gels were stained with 0.05% Coomassie blue in 12.5% aqueous trichloroacetic acid following the procedure of Chrambach et al. (17).

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate was done by the method of Shapiro, Vinuela, and Maizel (18) as modified by Weber and Osborne (19).

Cellulose acetate electrophoresis was done in 5 mM pH 7.4 KPO$_4$ buffer containing 10% glycerol and 1 mM 2-mercaptoethanol, and in Gelman high resolution barbital buffer.

### RESULTS

**Purification of α-Aminolevulinic Acid Synthase**

**Preparation of Cell Extract**—Frozen suspensions of *R. spheroides* were thawed and disrupted by use of the French press in 120-ml portions. The suspension of ruptured cells was diluted with an equal volume of 100 mM Tris-chloride buffer, pH 7.5, containing 0.5 mM EDTA, 10% glycerol, and 1 mM 2-mercaptoethanol. Unless otherwise indicated, all buffers used in subsequent steps contained all of these components at the stated concentrations.

**Protamine Sulfate Fractionation**—To the crude extract were added 33 ml of 4% protamine sulfate (neutralized with potassium hydroxide) quickly and with vigorous stirring. The mixture was immediately centrifuged at 30,000 × g for 15 min. The precipitate was set aside for recovery of coprecipitated enzyme and the supernatant was adjusted to 240 ml with 100 mM Tris-chloride buffer. This step appears to be critical in the purification. A yield of purified enzyme was only 10% of the total, most of the other units could be accounted for in various fractions not retained for purification.

**Ammonium Sulfate Fractionation**—Sixty grams of enzyme grade ammonium sulfate were added to the protamine sulfate supernatant. The solution was gently stirred for 20 min and centrifuged at 30,000 × g for 10 min. The supernatant fluid was discarded and the precipitate dissolved in a minimal volume of 10 mM Tris-chloride buffer, pH 7.5. The cell disruption, protamine sulfate, and ammonium sulfate treatments were repeated on 11 successive portions of cell suspension which were pooled for subsequent treatment. Speed seemed to be important during the early stages of the preparation. The dissolved ammonium sulfate precipitates were dialyzed against 4 1-liter volumes of the 10 mM Tris-chloride buffer at pH 7.5 for 48 hours. The dialyzed preparation was centrifuged at 100,000 × g for 60 min. The precipitate was discarded and supernatant liquid retained.

The yield at this point could be increased approximately 20% if the protamine sulfate precipitates were combined, resuspended in starting buffer containing one-half the concentration of protamine sulfate used initially, and centrifuged. The supernatant liquid was fractionated by the same procedure used for the original protamine sulfate supernatant fluid.

**DEAE-cellulose Chromatography**—The high speed centrifugation supernatant liquid was applied to a column of DEAE-cellulose (14 × 14 cm$^2$) which had been equilibrated with 10 mM Tris-chloride buffer at pH 7.5 without glycerol. The column was then washed with 400 ml of the same buffer following which the enzyme was eluted with a linear gradient of KC1 going from 100 to 200 mM all in 10 mM Tris-chloride buffer. The flow rate was 65 ml per hour and 20-ml fractions were collected. The most active fractions were combined, concentrated to 20 ml on the Diaflo UM 20E membrane, and dialyzed against 2 1-liter volumes of 10 mM potassium phosphate buffer, pH 6.8, for 24 hours.

**Calcium Phosphate Fractionation**—The dialyzed protein was applied to a column of calcium phosphate gel (20 cm × 14 cm$^2$) which had been equilibrated with 10 mM potassium phosphate buffer, pH 6.8. The column was washed with 200 ml of the same buffer following which the enzyme was eluted with a linear gradient of phosphate buffer going from 10 to 50 mM at pH 6.8. Elution with 500 mM potassium phosphate buffer at pH 6.8 did not release additional enzyme. The tubes of highest specific activity were pooled, concentrated (to 25 ml), and dialyzed against 1 liter of 5 mM potassium phosphate buffer at pH 7.4 for 48 hours.

**Hydroxylapatite Fractionation, pH 7.4**—The dialyzed fraction was applied to a column, 6 cm × 7 cm$^2$, of hydroxylapatite which had been equilibrated with 5 mM potassium phosphate buffer at pH 7.4. The column was washed with 100 ml of this buffer and the enzyme was eluted with a linear gradient of potassium phosphate buffer, pH 7.4, from 5 to 60 mM. The flow rate was 15 ml per hour and 10-ml fractions were collected. Potassium phosphate at 500 mM did not elute additional enzyme from this column. Fractions of constant specific activity were combined and concentrated on the UM 20E membrane.

The purification is summarized in Table I. Although the yield of purified enzyme was only 10% of the total, most of the other units could be accounted for in various fractions not retained for purification.

### Table I

**Purification of α-aminolevulinic acid synthase from *R. spheroides***

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Purification Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4,600</td>
<td>100</td>
<td>0.10</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>4,200</td>
<td>100</td>
<td>0.52</td>
<td>10</td>
<td>91</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>4,000</td>
<td>60</td>
<td>0.61</td>
<td>50</td>
<td>87</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>20</td>
<td>14</td>
<td>50</td>
<td>50</td>
<td>59</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>20</td>
<td>15</td>
<td>87</td>
<td>78</td>
<td>35</td>
</tr>
<tr>
<td>Hydroxylapatite, pH 7.4</td>
<td>20</td>
<td>7.5</td>
<td>470</td>
<td>130</td>
<td>10</td>
</tr>
</tbody>
</table>

* a After dilution. Eleven batches combined.

* b Including activity recovered from the protamine sulfate precipitates.
Properties of Purified δ-Aminolevulinic Acid Synthase

Estimation of Purity—The purified δ-aminolevulinic acid synthase, along with fractions obtained during purification, was examined by polyacrylamide gel electrophoresis in the discontinuous pH 9.8 system (16). The preparation having 100 units per mg showed one major band and two minor bands. In addition we generally noted a diffuse, lightly stained area trailing the major band. The same preparation, in the presence of 0.1% sodium dodecyl sulfate, showed only one band of significant intensity when examined by polyacrylamide gel electrophoresis. This suggests that aggregation of δ-aminolevulinic acid synthase could be responsible for the minor bands observed in the absence of sodium dodecyl sulfate.

Thus far it has not been possible to prove unequivocally that the major protein band on polyacrylamide gel electrophoresis is in fact δ-aminolevulinic acid synthase, since it is not possible to recover active enzyme following this step. It is, however, possible to recover active δ-aminolevulinic acid synthase following electrophoresis on cellulose acetate at pH 6.9 or 8.9. In such cases, the activity corresponds in position to the only stainable protein band. We, therefore, conclude on the basis of these experiments that the major band obtained on polyacrylamide gel electrophoresis is δ-aminolevulinic acid synthase.

Molecular Weight—Polyacrylamide gel electrophoresis with sodium dodecyl sulfate was used to determine the molecular weight of the monomeric form of δ-aminolevulinic acid synthase (18, 19). The electrophoretic mobilities of δ-aminolevulinic acid synthase and proteins of established molecular weight are shown in Fig. 1. The calculated molecular weight of 57,000 ± 5,000 obtained by this method is in reasonable agreement with that obtained by ourselves and by Kikuchi's group with gel filtration on Sephadex G-200 (20).

Stability of δ-Aminolevulinic Acid Synthase—The purified enzyme is stable when stored at -15° in 50 mM phosphate buffer at pH 6.8 in the presence of 10% glycerol and 1 mM 2-mercaptoethanol for several months. Little or no loss in activity is observed when samples are repeatedly frozen and thawed. When stored at 4° in the same buffer at least 90% of the activity is retained after 2 weeks. This contrasts with crude enzyme preparations in which an initial increase in activity, followed by a rapid loss to about 25% of the original activity after 24 hours at 4°, is observed. At 37° in the above buffer the enzyme retained about 80% activity after 60 min while in the same buffer lacking glycerol and mercaptoethanol it only retained about 40% activity after 60 min.

pH Optimum—Potassium phosphate and Tris-chloride buffers were used where appropriate, and overlap was achieved between pH 7.2 and 7.6. The pH optimum was in the region 7.4 to 7.6 with slightly higher activity in phosphate buffer.

Effect of Potassium Ferricyanide and Dithiothreitol on Purified Enzyme—Both potassium ferricyanide and dithiothreitol strongly affect the activity of crude preparations of δ-aminolevulinic acid synthase (see below). Neither compound produced the same effect on the activity of purified enzyme. The purified enzyme was slightly inhibited by potassium ferricyanide, whereas, it was activated up to 2-fold by dithiothreitol, both at the same concentrations used in studies on crude preparations.

Inhibition of δ-Aminolevulinic Acid Synthase by Hemin—Previous reports on partially purified δ-aminolevulinic acid synthase have documented that hemin exerts a strong inhibitory effect (6, 7, 21). That the effect of hemin is not indirect, as is the case with oxidizing and reducing compounds, is shown in Table II. The purified enzyme is more sensitive to hemin than previous reports indicated (6).

Two series of experiments were carried out to examine the reversibility of inhibition by hemin. In one series, δ-aminolevulinic acid synthase and hemin were mixed in constant proportion and initially incubated at 4° for 10 min. At that time, graded amounts of rabbit apohemopexin, a specific hemin binding β-globulin (22), were added to the hemin-δ-aminolevulinic acid synthase mixtures and a second 10-min preliminary incubation at 4° was performed. The results showed that apohemopexin caused a reversal of the hemin inhibition to the extent that would be predicted from the change in concentration of hemin. The change in concentration of hemin is calculated on the basis that apohemopexin binds hemin on a 1:1 molar ratio.

Experiments were also conducted to see if the inhibition of δ-aminolevulinic acid synthase by hemin could be reversed by diluting out the hemin. To do this, hemin was added directly to purified δ-aminolevulinic acid synthase at a final concentration of 100 μM. After 10 min of incubation at 4°, samples of varying size were withdrawn and added to the assay mixture. A parallel control experiment was carried out in which case no hemin was added to the purified enzyme. As can be seen in

<table>
<thead>
<tr>
<th>Hemin concentration (μM)</th>
<th>δ-Aminolevulinic acid synthase activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>0.01</td>
<td>63</td>
</tr>
<tr>
<td>0.1</td>
<td>61</td>
</tr>
<tr>
<td>0.5</td>
<td>54</td>
</tr>
<tr>
<td>1.0</td>
<td>47</td>
</tr>
<tr>
<td>3.0</td>
<td>39</td>
</tr>
<tr>
<td>5.0</td>
<td>30</td>
</tr>
</tbody>
</table>

Fig. 1. Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. The molecular weight of marker proteins is plotted on a semilogarithmic scale against the migration distance from the top of the gel relative to hemoglobin (18, 19). 1, serum albumin; 2, catalase; 3, δ-aminolevulinic acid synthase, 4, pepsin, 5, hemoglobin.
TABLE III
Reversal of hemin inhibition of δ-aminolevulinic acid synthase by dilution

Hemin was added to 1 ml of purified δ-aminolevulinic acid synthase in 0.05 M Tris, pH 7.5, at 4°. After 10 min of preliminary incubation aliquots were removed and added to the standard assay mixture also at 4°. The tubes were then incubated at 37°. A parallel experiment was run in which water was substituted for hemin.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Without hemin</th>
<th>With hemin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total units</td>
<td>units/mg</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>62</td>
</tr>
<tr>
<td>10</td>
<td>57</td>
<td>63</td>
</tr>
<tr>
<td>20</td>
<td>120</td>
<td>67</td>
</tr>
<tr>
<td>50</td>
<td>314</td>
<td>69</td>
</tr>
<tr>
<td>100</td>
<td>576</td>
<td>62</td>
</tr>
<tr>
<td>150</td>
<td>890</td>
<td>67</td>
</tr>
<tr>
<td>200</td>
<td>1100</td>
<td>61</td>
</tr>
<tr>
<td>300</td>
<td>1420</td>
<td>53</td>
</tr>
</tbody>
</table>

Table III, the activity of δ-aminolevulinic acid synthase at high dilution is greater than at low dilution indicating that enzyme bound hemin is in equilibrium with free hemin. In the absence of hemin, the specific activity is constant and product formation linear up to 200 μl of added enzyme. When the results of these experiments are compared with direct hemin inhibition experiments (Table II), it is apparent that, although hemin inhibition is reversed by dilution, the reversal is not complete.

**Kinetic Studies**—In a series of preliminary kinetic studies, K \text{m} \text{ values for glycine and succinyl-CoA were determined and found to be 10 mM and 25 μM, respectively. In these experiments all components of the assay system were saturating except for the one being varied. In an attempt to derive more information from the kinetic analysis a series of experiments modeled after the method of Cleland (23) were run. (a) Glycine variable at different succinyl-CoA concentrations and saturating pyridoxal phosphate: Plots of 1/v versus 1/[s] revealed a family of straight lines converging above the ordinate. (b) Succinyl-CoA variable at different glycine concentrations and saturating pyridoxal phosphate: Plots of 1/v versus 1/[s] revealed a family of straight lines converging above the ordinate. (c) Pyridoxal phosphate variable at different levels of glycine or succinyl-CoA with succinyl-CoA or glycine saturating: Plots of 1/v versus 1/[s] were not straight lines, nor did they appear to have any recognizable shape. On the other hand when both glycine and succinyl-CoA were saturating, the plotted data fit recognizable patterns. Plots of v versus [s] appeared hyperbolic. Double reciprocal plots, however, were not linear. The hyperbolic appearance stemmed from the compression of the substrate scale required to get the data on a single graph. The data are best illustrated in plots of v versus log [s] (Fig. 2).

Inhibition studies were carried out with hemin and pyridoxal phosphate, at saturating glycine and succinyl-CoA. Inhibition was observed at all pyridoxal phosphate concentrations, and a v versus log [s] plot had the same shape as similar plots in the absence of hemin.

**Crude Enzyme Preparations**

In the early work with δ-aminolevulinic acid synthase, it was noted that there was sometimes considerable fluctuation in the enzyme activity of crude bacterial extracts. This had nothing to do with induction or repression of δ-aminolevulinic acid synthase, but was caused by variations in handling the bacterial suspensions following harvest (8, 9, 11).

The effect was traced to atmospheric oxygen. The phenomenon was studied by testing the effect of various oxidizing and reducing agents upon crude enzyme preparations. Potassium ferricyanide was the oxidant studied in most detail, while dithiothreitol was the most studied reductant. The results of typical experiments are summarized in Table IV. That the effect of oxidizing and reducing agents is indirect is attested by the fact that the effect is not observed with purified δ-aminolevulinic acid synthase (see above).

During studies of the activation of δ-aminolevulinic acid synthase by potassium ferricyanide, it was observed that the degree of activation was markedly affected by the concentration of enzyme used in the assay. We, therefore, examined the yields of δ-aminolevulinic acid over wide ranges of enzyme con-
were added to a series of assay tubes. After 30 min at 37°C the re-
mixture, or with dithiothreitol at 100 

Parently contradictory results appear to arise from the fact that oxidation-reduction state of the enzyme extract. Their ap-
tained by both of these groups of workers by changing the activator. To a large extent, we can duplicate the results ob-
tively low enzyme concentrations found evidence for an enzyme present in the extract while Marriott et al. (8) working at rela-
tively high enzyme concentrations concluded that an inhibitory substance was present in the extract while Marriott et al. (8) working with relatively high en-
zyme concentrations observed a decrease in the rate of d-aminolevulinic acid synthase in crude extracts, but under different experimental conditions. Tuboi et al. (9) working with relatively high en-
zyme concentrations concluded that an inhibitory substance was present in the extract while Marriott et al. (8) working at rela-
tively low enzyme concentrations found evidence for an enzyme activator. To a large extent, we can duplicate the results ob-
tained by both of these groups of workers by changing the oxidation-reduction state of the enzyme extract. Their ap-
parently contradictory results appear to arise from the fact that they are looking at different aspects of the same phenomenon.

**DISCUSSION**

Previous attempts at the purification of δ-aminolevulinic acid synthase from *R. spheroides* have met with only modest success. In retrospect, it is possible to recognize some of the obstacles that delayed progress.

One special problem involved the recognition of the dual role of sulfhydryl compounds. Attempts to stabilize the enzyme by adding sulfhydryl reagents were not successful because these compounds promote the inhibition of the enzyme in crude ex-
tracts. Once the enzyme is purified (even partially), the ad-
dition of sulfhydryl compounds no longer inhibits the enzyme, and in fact they stabilize, or re-activate the enzyme, or both. Certainly once δ-aminolevulinic acid synthase is activated with potassium ferricyanide, activity is lost much more rapidly during storage than in extracts not subjected to activation.

The question, of course, remains as to an explanation for this oxidation-reduction sensitivity. It could be an artifact manifest only in broken cell preparations and thus be of no physiological importance. Another possibility is that the oxidation reduction sensitivity could be caused by the existence of an oxidation-reduction material that functions in a regulatory capacity. The regulation of pigment synthesis in *R. spheroides* has been discussed for a number of years in terms of the existence of a regulatory substance, other than hemin, sensitive to the oxidation-reduction state of the cell (24-26). The fact that there is evidence for natural inhibitors of δ-aminolevulinic acid synthase in preparations other than those from *R. spheroides* (1, 7, 10) suggests that perhaps their presence is more than coincidence.

It is noteworthy that neither succinyl-CoA nor glycine produced sigmoidal r versus [s] curves "expected" of allosteric en-
zymes. Indeed, only pyridoxal phosphate generated kinetic plots that were not hyperbolic. The results obtained with pyridoxal phosphate suggest caution in making statements regarding *Km* values for this substance (20).

The apparent molecular weight of 57,000 observed for δ-
aminolevulinic acid synthase and the fact that it is apparently the same in the presence and absence of sodium dodecyl sulfate is further evidence that this enzyme does not fit the general model of allosteric enzymes. It may be that δ-aminolevulinic acid synthase is an exception to the model. Alternatively, maybe conditions will yet be found under which the enzyme behaves according to the model. The possibility that regulatory sub-
units might have been lost during purification appears unlikely since hemin is active as an effector even with the highly purified enzyme.

In contrast with δ-aminolevulinic acid synthase from *R. spheroides*, a preliminary communication on the enzyme from reticulocytes indicates that the enzyme from that source has an apparent molecular weight of 200,000 (27). δ-Aminolevulinic acid synthase from that source differs further from the *R. spheroides* enzyme in that it is not sensitive to the addition of pyridoxal phosphate; it still shows 60% of maximum activity.

**REFERENCES**


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**Fig. 3. δ-Aminolevulinic acid formation at different enzyme concentrations.** Appropriate volume of a single enzyme extract were added to a series of assay tubes. After 30 min at 37°C the reaction was stopped and aliquots were removed for δ-aminolevulinic acid determination. Samples of the same extract were treated with potassium ferricyanide at a final concentration of 500 μM, or with dithiothreitol at 100 μM. H2O control (○), potassium ferricyanide (□), dithiothreitol (△).
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