Human Amidophosphoribosyltransferase

AN OXYGEN-SENSITIVE IRON-SULFUR PROTEIN*

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Glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase (EC 2.4.2.14), amidophosphoribosyltransferase, was partially purified from human placenta. Upon exposure to oxygen, both the glutamine and ammonia activities were lost in parallel. Inactivation by oxygen increased as the temperature of incubation rose and the partial pressure of oxygen increased. Molecular oxygen rather than a radical derivative was responsible for inactivation since scavengers of oxygen radicals did not protect against inactivation. AMP, GMP, PP-ribose-P, and inorganic phosphate partially protected both the glutamine and ammonia activities from inactivation by oxygen. Incubation with 1,10-orthophenanthroline, but not 1,7-metaphenanthroline or tiron, led to inactivation of amidophosphoribosyltransferase. Both the 1,10-orthophenanthroline- and oxygen-inactivated enzymes could be reconstituted by incubation with ferrous iron and inorganic sulfide in the presence of dithiothreitol under anaerobic conditions. The iron requirement could not be replaced by zinc, copper, cobalt, nickel, magnesium, or calcium. The sulfide requirement could not be replaced by higher concentrations of dithiothreitol. It is concluded from these studies that human amidophosphoribosyltransferase is an iron-sulfur protein and oxidation of this structure may be responsible for the marked lability of this enzyme in vitro.

From B. subtilis was an iron-sulfur protein and presented evidence indicating that oxidation of the iron-sulfur center of the enzyme was responsible for inactivation following exposure to oxygen. However, this explanation may not be applicable to all forms of amidophosphoribosyltransferase since the pigeon liver enzyme, which is also unstable in vitro (11), is reported not to contain sulfide (6). The amidophosphoribosyltransferase isolated from Escherichia coli is probably different from both the pigeon liver and B. subtilis enzymes in this it does not contain iron (8), a metal found in the avian (5, 6) and B. subtilis (7) proteins.

Human amidophosphoribosyltransferase, like that from bacteria and avian liver, is also unstable in vitro and this property of the enzyme has retarded our attempts to purify the protein. Since amidophosphoribosyltransferase from different sources appears to be heterogeneous in structure, we have initiated studies to explain the instability of the human enzyme in vitro. In this communication we present findings which indicate that human amidophosphoribosyltransferase is inactivated by molecular oxygen and report data which suggest, too, is an iron-sulfur protein.

EXPERIMENTAL PROCEDURES

Materials

L-[14C]Glutamine (49 mCi/mmol) and L-[35S]cysteine (179 mCi/mmol) were purchased from Amersham/Searle. The glutamine was purified as previously described (12) and the cysteine was used without further purification.

Sodium salts of PP-ribose-P, AMP, and GMP, as well as catalase (300 units/mg), L-histidine, and inosine were purchased from Sigma. Dithiothreitol was purchased from Liestal/Schweiz. 1,10-Orthophenanthroline was purchased from Sigma and 1,7-metaphenanthroline was from Frederick Smith. Sodium sulfide was purchased from Fisher and ferrous ammonium sulfate was from J. T. Baker. Ferrous ammonium sulfate was dissolved in 1 N HCl, gassed with nitrogen for 2 h to remove sulfide, and stored overnight in an anaerobic chamber. Before use, the solution was neutralized and 10 mM dithiothreitol was added. Azaserine was purchased from Calbiochem. Superoxide dismutase, purified from bovine erythrocytes, was a kind gift from Dr. Irwin Fridovich of the Department of Biochemistry, Duke University Medical Center. CM-Sephadex and DE52 were purchased from Whatman and Sephadex G-25 (fine) was from Pharmacia.

Glass tubes sealed with rubber stoppers, a product of Becton, Dickinson Co., were used for incubating enzyme samples under oxygen or nitrogen. For experiments performed under anaerobic conditions, a chamber (65 × 32 × 40 inches) equipped with platinum heating elements was filled with 95% nitrogen, 5% hydrogen (Coy Laboratory Products). Cylinders of 100% oxygen, 100% nitrogen, and 95% nitrogen, 5% hydrogen were obtained from National Welders.

Methods

Enzyme Purification—Fresh human placenta was homogenized in a Waring Blender for 90 s in 15 mM KP buffer, pH 6.0, which

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‡ The abbreviation used is: PP-ribose-P, 5-phosphoribosyl-1-pyrophosphate.
As depicted in Fig. 2, the plot of percentage inactivation versus atmospheres of oxygen was hyperbolic with saturation occurring at approximately 1 atm of oxygen. No apparatus was readily available for performing experiments under hyperbaric conditions so it was not possible to determine experimentally the partial pressure of oxygen which was saturating perbaric conditions so it was not possible to determine experimentally the partial pressure of oxygen which was saturating.

In addition to temperature, the rate of inactivation was dependent upon the partial pressure of oxygen to which the enzyme was exposed (Fig. 2). Conditions were selected for this experiment so that approximately 50% of the catalytic activity was lost during the period of exposure to the highest oxygen concentration (1.0 atm for 25 min at 37°C). Had the incubation been continued for a longer time or performed at a higher temperature, all of the catalytic activity would have been lost. Percentage inactivation was determined in room air by the assays described above.

RESULTS

Oxygen Inactivation of Amidophosphoribosyltransferase—In Tris-HCl buffer, amidophosphoribosyltransferase was rapidly inactivated upon exposure to oxygen; glutamine and ammonia activities were lost in parallel (Fig. 1). There was no loss of glutamine or ammonia activity during incubation for 1 h at 37°C under 100% nitrogen.

Enzyme and Protein Assays—(A) Glutamine utilization by amidophosphoribosyltransferase was quantified by determining the PP-ribose-P-dependent hydrolysis of [14C]glutamine (12). (B) Ammonia utilization by amidophosphoribosyltransferase was assayed by quantifying phosphoribosylamine production with [33S]cysteine (13). (C) Protein was determined by the method of Lowry et al. (14).

Oxygen Inactivation of Amidophosphoribosyltransferase—One milliliter of the enzyme preparation was passed through a Sephacryl G-25 column (1 x 21 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.4 at 25°C. The peak protein fractions were pooled. This and all subsequent steps were performed at 4°C. Two hundred microliters of enzyme plus ligand were placed in glass tubes (1.3 x 9.5 cm) and the tubes were then capped with an air-tight rubber stopper. Two 21-gauge needles were inserted into the rubber stopper and the contents of the tube were gassed with 100% oxygen or 100% nitrogen. One-half liter of gas was flushed into the tube over a 1-min period through one needle and evacuated through a second needle. At the end of the gas exchange, both needles were removed and the sealed tubes were incubated for 0 to 60 min at temperatures ranging from 4 to 45°C. Following this incubation, the tubes were opened and enzyme catalytic activity was determined in room air by the assays described above.

1,10-Orthophenanthroline Inactivation of Amidophosphoribosyltransferase—The enzyme preparation was incubated with 20 mM 1,10-orthophenanthroline in Tris-HCl buffer, pH 7.4 at 37°C. The peak protein fractions were pooled, concentrated 10- to 15-fold in an Amicon ultrafiltration cell, and stored at -70°C. The specific activity of the enzyme preparation used in these studies was 396 nmol/h/mg. Glutaminase and nucleotidase activity were not detectable under the assay conditions described below.

Protection Against Oxygen Inactivation—A number of concentrations of superoxide dismutase (5 μg/ml), catalase (500 μg/ml), l-histidine (5 mM), glucose (5 mM), and inosine (5 mM), which are effective in scavenging O2-, O(2-)Ag, OH-, and H2O2 (15), did not significantly prolong the t1/2 of amidophosphoribosyltransferase during the exposure to oxygen (Table II). These results suggest that molecular oxygen, rather than an oxygen radical, is responsible for inactivation.
ligands were found to prolong the $t_{1/2}$ of amidophosphoribosyltransferase activity. Data from these experiments are presented in Tables III and IV. Both the glutamine and ammonia activities were more resistant to oxygen inactivation in the presence of PP-ribose-P, inorganic phosphate, and purine ribonucleotides, allosteric effectors of human amidophosphoribosyltransferase (12, 16, 17). The protective effect of these ligands was concentration-dependent and the values presented in Tables III and IV were obtained with the concentrations that provided maximal protection.

Glutamine and ammonia, alone or in combination, did not protect against oxygen inactivation in the absence of PP-ribose-P (Table IV). When these substrates were combined with PP-ribose-P, the $t_{1/2}$ was prolonged, but the protection provided by the combined substrates was no greater than that afforded by PP-ribose-P alone. However, azaserine, a glutamine analogue, did provide protection. Unlike glutamine, azaserine binds irreversibly to amidophosphoribosyltransferase and this interaction between the enzyme and azaserine is enhanced by simultaneous incubation with PP-ribose-P (18, 19). As shown in Table IV, azaserine alone at high concentrations prolonged the half-life of amidophosphoribosyltransferase catalytic activity and this effect was enhanced in the presence of PP-ribose-P. These experiments were performed with an enzyme preparation that had been passed through a Sephadex G-25 column before incubation under oxygen. Consequently, the effect of azaserine plus PP-ribose-P was the result of azaserine irreversibly bound to the enzyme and not simply the protective effect of PP-ribose-P. Further proof that azaserine was bound to the enzyme was demonstrated by the failure to detect glutamine activity in the effluent from the G-25 column. Since ammonia activity of human amidophosphoribosyltransferase is not inhibited by azaserine (19), it was possible to demonstrate that this activity was protected from oxygen inactivation by azaserine.

In addition to the effect of ligands, substrates, and substrate analogues, dithiothreitol and β-mercaptoethanol were found to protect amidophosphoribosyltransferase from oxygen inactivation. Dithiothreitol (5 mM) prolonged the half-life approximately 3-fold and 60% of the enzyme activity was protected from inactivation. Dithiothreitol (5 mM) prolonged the half-life approximately 1.5-fold. Both the glutamine and ammonia activities were comparably protected.

**Orthophenanthroline Inactivation—** Amidophosphoribosyltransferase isolated from chicken liver (5), pigeon liver (6), and *B. subtilis* (7) has been reported to contain iron, whereas that from *Escherichia coli* is thought not to contain iron (8). Those forms of the enzyme demonstrated to contain iron are inhibited by 1,10-orthophenanthroline but not by 1,7-meta phenanthroline (5–7). As shown in Fig. 3, human amidophosphoribosyltransferase catalytic activity was inhibited by 1,10-orthophenanthroline but not by 1,7-metaphenanthroline. Al-
Amidophosphoribosyltransferase: an O$_2$-sensitive Fe-S Enzyme

Experimental design and method of data presentation are explained in the legend of Table II. For the experiments with NH$_4$Cl and glutamine these substrates were added after the enzyme was passed through the Sephadex G-25 column and were present at the concentration indicated during the incubation under oxygen. For the experiments with azaserine, this substrate analogue was incubated with the enzyme for 15 min at 37°C at the concentration indicated before passage through the Sephadex G-25 column and was not added to the enzyme during the incubation under oxygen.

\[
\begin{array}{ccc}
\text{Substrates} & \text{Substrate Control t$_{1/2}$} & \text{Glutamine activity} & \text{Ammonia activity} \\
\text{Gln, } 5 \text{ mM} & 1.06 & 1.06 \\
\text{NH$_4$Cl, } 150 \text{ mM} & 0.97 & 1.17 \\
\text{Gln, } 5 \text{ mM + NH$_4$Cl, } 150 \text{ mM} & 1.00 & 1.04 \\
\text{Gln, } 5 \text{ mM + PP-ribose-P, } 5 \text{ mM} & 4.67 & 5.20 \\
\text{NH$_4$Cl, } 150 \text{ mM + PP-ribose-P, } 5 \text{ mM} & 5.00 & 9.00 \\
\text{Azaserine, } 40 \text{ mM} & 3.18 & 3.50 \\
\text{Azaserine, } 40 \text{ mM + PP-ribose-P, } 5 \text{ mM} & \text{—} & 16.63 \\
\end{array}
\]

The concentrations of l,lO-orthophenanthroline (M) and other metal chelators. The partially purified enzyme was first incubated with l,lO-orthophenanthroline-inactivated enzyme on six occasions and the results of each experiment are presented. Six separate experiments were performed and the results of each are presented.

The experimental design is the same as that described in the legend to Table V. Three separate experiments were performed and the results of each are presented.

Reconstitution of 1,1O-orthophenanthroline-inactivated amidophosphoribosyltransferase

The enzyme was incubated with orthophenanthroline and passed through a Sephadex G-25 column as described under "Methods." Six separate experiments were performed and results of each are presented. The enzyme was incubated with 10 mM diithiothreitol, 1.0 mM sodium sulfide, 0.250 mM ferrous ammonium sulfate, or a combination of these agents for 20 to 24 h at 25°C in an anaerobic chamber and the activity was measured as described under "Methods."

\[
\begin{array}{cccccc}
\text{Additions} & \text{Enzyme activity in Experiment}^a & 1 & 2 & 3 & 4 & 5 \\
\hline
\text{None} & - & 59 & 60 & 58 & 79 & 23 & 24 \\
\text{Dithiothreitol} & - & 83 & 93 & 117 & 80 & 67 & 52 \\
\text{Dithiothreitol + S}^2- & - & 97 & 112 & 113 & 91 & 74 & 56 \\
\text{Dithiothreitol + Fe}^{3+} & - & 133 & 144 & 144 & 109 & 113 & 86 \\
\text{Dithiothreitol + Fe}^{3+} + S^2- & - & 169 & 155 & 182 & 151 & 131 & 112 \\
\end{array}
\]

\^ First line values are activity at time zero. All other values are activity after incubation anaerobically.

<table>
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<th>Additions</th>
<th>Enzyme activity in Experiment$^a$</th>
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<th>3</th>
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<td>72</td>
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<td>95</td>
<td>96</td>
<td>93</td>
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</tbody>
</table>

\^ First line values are activity at time zero. All other values are activity after incubation anaerobically.

were obtained with this standard set of conditions.

Reconstitution experiments were performed with the orthophenanthroline-inactivated enzyme on six occasions and the results of each experiment are presented in Table V. Incubation with 10 mM diithiothreitol alone resulted in a mean restoration of 12.5 ± 8.0% (1 S.D.) of the amidophosphoribosyltransferase activity. Increasing the diithiothreitol concentration above 10 mM did not produce a greater percentage of reconstitution. Addition of sulfide to diithiothreitol increased the percentage of reconstitution slightly, mean of 18.9 ± 8.0%. Addition of iron to diithiothreitol increased the percentage of reconstitution to 28.7 ± 8.8% (p < 0.001 when compared to diithiothreitol alone). The combination of iron plus sulfide and diithiothreitol produced the greatest percentage of reconstitution, mean of 40.6 ± 7.5% (p < 0.001 when compared to diithiothreitol plus iron alone). The requirement for iron could not be replaced by nickel, cobalt, copper, zinc, magnesium, or calcium. In the absence of diithiothreitol, neither ferrous nor ferric iron produced significant reconstitution. The effect of sulfide could not be attributed to sulfhydryl reduction because all samples contained 10 mM diithiothreitol and higher concentrations of diithiothreitol did not replace the requirement for sulfide.

Three experiments were performed with the oxygen-inactivated enzyme and the results of these experiments are presented in Table VI. Dithiothreitol alone produced a mean percentage reconstitution of 7.6 ± 2.0%; diithiothreitol plus sulfide, 11.2 ± 3.0%; and diithiothreitol plus iron, 8.0 ± 1.8%. The combination of iron plus sulfide and diithiothreitol produced the greatest percentage reconstitution, mean of 18.7 ±
Amidophosphoribosyltransferase: an O2-sensitive Fe-S Enzyme

2.3% (p < 0.01 when compared to dithiothreitol alone). For the oxygen-inactivated enzyme, the percentage of reconstitution was less than that obtained for the orthophenanthroline-inactivated enzyme but the characteristics of reactivation were similar in that maximal reconstitution was obtained with iron plus sulfide in the presence of dithiothreitol. However, in the case of the orthophenanthroline-inactivated enzyme, there was a significant effect of iron alone on reconstitution of catalytic activity, whereas the oxygen-inactivated enzyme demonstrated only a minimal effect of iron alone on reconstitution.

DISCUSSION

Results presented here demonstrate that human amidophosphoribosyltransferase is inactivated upon exposure to oxygen. This may explain the lability of catalytic activity in vitro and account for some of the difficulty experienced in attempting to purify human amidophosphoribosyltransferase.

Switzer and colleagues have shown that amidophosphoribosyltransferase purified from B. subtilis contains 3 mol of iron and 2 mol of sulfide/mol of protein, and they have reported that oxidation of the unique iron-sulfur center of this protein is responsible for oxygen inactivation (7). Studies with other iron-sulfur proteins also suggest that oxygen inactivation is related to oxidation of sulfide or FeS, or both, with concomitant disruption of the iron-sulfur center (20, 21).

If human amidophosphoribosyltransferase were demonstrated to contain iron and sulfide, this might explain the sensitivity of this enzyme to oxygen inactivation. Results of the orthophenanthroline experiments indicate that human amidophosphoribosyltransferase contains a heavy metal, and the reconstitution studies suggest that this metal is iron, possibly FeS.

Reconstitution of the orthophenanthroline-inactivated and oxygen-inactivated enzyme suggests that human amidophosphoribosyltransferase contains sulfide as well as iron. For the oxygen-inactivated enzyme incubation with iron alone or sulfide alone had little effect on catalytic activity, but incubation with the combination of iron plus sulfide restored a significant amount of catalytic activity. These results demonstrate a requirement for both iron and sulfide for reconstitution of catalytic activity. For the orthophenanthroline-inactivated enzyme, incubation with iron alone was partially effective in restoring activity, incubation with sulfide alone had little effect, and incubation with the combination of iron plus sulfide was more effective than iron alone. It is not surprising that iron alone was partially effective in restoring activity to the orthophenanthroline-inactivated enzyme since removal of iron from other proteins with chelating agents does not always lead to a loss of the sulfide. In some cases, sulfide remains in the protein but in a higher state of oxidation (21, 22). Under these conditions, dithiothreitol can partially reduce the oxidized sulfide and relieve the absolute requirement for an exogenous source of sulfide (21). Thus, the reconstitution studies presented here demonstrate that iron, probably the ferrous salt, and inorganic sulfide are required for restoration of catalytic activity of human amidophosphoribosyltransferase following oxygen and orthophenanthroline inactivation. These findings suggest this enzyme, like that from B. subtilis, is also an iron-sulfur protein.

A still unresolved question is the role of the iron-sulfur center in the physiological function of amidophosphoribosyltransferase. This enzyme catalyzes two separate but related reactions: glutamine hydrolysis and synthesis of phosphoribosylamine from NH3. Moreover, reconstitution studies demonstrate that incubation with iron and sulfide leads to a restoration of the latter, as well as former, activity of amidophosphoribosyltransferase. These findings suggest that the iron-sulfur center plays an important role in the utilization of NH3 for phosphoribosylamine synthesis.

Another potential function of the iron-sulfur center of amidophosphoribosyltransferase is to act as a sensor of the oxidation-reduction potential in the cell. Ruzicka and Beinert have presented data which suggest that aconitase, a high potential iron-sulfur protein, may function as a sensor of the oxidation-reduction state of the cell and postulate that reversible reduction and oxidation of the iron-sulfur cluster of other enzymes may be a general principle used in nature to regulate the activity of some enzyme systems (24). Reversible oxidation and reduction of the iron-sulfur center of amidophosphoribosyltransferase could potentially lead to changes in the activity of this enzyme, and this might provide yet another mechanism for the control of amidophosphoribosyltransferase activity. In addition, this type of reaction might provide a link between amidophosphoribosyltransferase and other iron-sulfur proteins since all studies reported to date indicate that iron-sulfur proteins function as electron carriers (25–29) or participate in oxidation-reduction reactions (29).

REFERENCES


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