Reaction of *Bacillus subtilis* Glutamine Phosphoribosylpyrophosphate Amidotransferase with Oxygen: Chemistry and Regulation by Ligands†

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ABSTRACT: The inactivation of glutamine phosphoribosylpyrophosphate amidotransferase by reaction of its iron–sulfur center with O_2 is believed to be a physiologically important mode of regulation of this enzyme in *Bacillus subtilis* cells in the stationary phase of growth. Chemical and physical changes accompanying oxidation of the purified enzyme by O_2 were studied. The iron of the 4Fe–4S center was oxidized to enzyme-bound high-spin Fe^{3+}; the S^{2-} was oxidized to a mixture of S^0 bound as thiocystine and unidentified products. The oxidant appeared to be O_2, rather than peroxide, superoxide, hydroxyl radical, or singlet oxygen. Gross physical changes in the oxidized enzyme were shown by its aggregation, decreased solubility, and altered circular dichroic spectrum. Experimental variables affecting the rate of oxidative inactivation were described; the most important of these was modulation of rates of inactivation by the allosteric inhibitors AMP, ADP, GMP, and GDP and by the substrate P-Rib-PP. AMP was a potent stabilizer, whose effect was antagonized by P-Rib-PP. The other nucleotides, either acting singly or acting as synergistic pairs, were destabilizers and able to antagonize stabilization by AMP. The results are discussed in terms of the regulation of the stability of amidotransferase and its degradation in vivo.

The first enzyme of purine biosynthesis, glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14), hereafter called "amidotransferase", is regulated in *Bacillus subtilis* not only by repression (Nishikawa et al., 1967) and end-product inhibition (Meyer & Switzer, 1979) but also by oxygen-dependent inactivation in cells in the stationary phase of growth (Turnbough & Switzer, 1975a). The nature of the oxygen-dependent inactivation was clarified by the discovery that amidotransferase is inactivated by reaction with oxygen in cell-free extracts (Turnbough & Switzer, 1975b) and the subsequent demonstration that pure amidotransferase is an oxygen-labile iron–sulfur protein (Wong et al., 1977; Averill et al., 1980). Inactivation of amidotransferase results from reaction of oxygen with the iron–sulfur center. Inactivation is accompanied by bleaching of the iron–sulfur chromophore (Wong et al., 1977), loss of inorganic sulfide (Switzer et al., 1979a,b), and conversion of the iron from a form assaying as Fe^{2+} to Fe^{3+} (Switzer et al., 1979a,b). No "inactivating enzyme" is required for this process. A clue to the regulation of the inactivation of amidotransferase was provided by the observation that the rate of inactivation of the enzyme in crude extracts was strongly affected by allosteric ligands (Turnbough & Switzer, 1975b). This paper presents the results of a complete study of the chemical and physical changes accompanying oxygen-dependent inactivation of highly purified amidotransferase and of the modulation of the rate of inactivation by substrates, allosteric ligands, and other potential regulators. A tentative model for the regulation of the stability of amidotransferase to O_2 in vivo has been deduced from the results.

Experimental Procedures

Materials. All amidotransferase samples used in this study were prepared by the method of Wong et al. (1981). Enzyme concentrations were determined by using an extinction coefficient ($E^{1\%}_{1cm}$) of 9.6 at 278 nm (Wong et al., 1977). *N*-[*ethyl*-^{14}C]Ethylmaleimide was purchased from New England Nuclear. Poly(ethylenimine) sheets were purchased from Brinkmann Instruments Inc. L-Glutamine, dithiothreitol, and dithioerythritol were purchased from Calbiochem-Behring Corp. All other nucleotides and crystalline ribonuclease A (RNase A),[1] type IIA, were obtained from Sigma Chemical Corp. Most nucleotides were of the highest purity available and, except as noted, were used without further purification.

Amidotransferase Assays. Amidotransferase activity was assayed as described in the preceding paper (Wong et al., 1981). Assays of the rate of inactivation of amidotransferase by O_2 were conducted in the following manner. A stream of dry O_2 was passed through three 250-mL filter flasks connected in series and filled with double-distilled water to humidify the O_2. The humidified oxygen was then passed via a 19-gauge needle through a stoppered serum vial and allowed to bubble through the reaction buffer for 5 min before the needle was withdrawn. For control experiments, argon-flushed buffers were used. All manipulations were performed in a 37 °C water bath. Essentially no loss of volume was obtained when the above humidification procedure was used. Without prior humidification, up to 50% of the solution volume was lost during bubbling. Five minutes of bubbling was shown to be sufficient time to saturate the solution with O_2, as judged by the constancy of the amidotransferase half-life at longer bubbling times.

Inactivation experiments were usually carried out in a 50 mM Tris-HCl, pH 7.9, 2.5 mM EGTA, and 10 mM $MgCl_2$ buffer at 37 °C. Biochemicals to be tested were added to this inactivation buffer either as solids or as concentrated solutions in 50 mM Tris-HCl, pH 7.9. The pH of the resultant mixture was readjusted to 7.9 when necessary.

Zero time for the inactivation is defined as the addition of concentrated stock amidotransferase (5–10 μL) into the preoxygenated reaction mixture (2000 μL final volume). The final concentration of amidotransferase in most studies was 20 μg/mL. Aliquots of 50–100 μL were removed from the

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[1] Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; P-Rib-PP, 5-phospho-α-D-ribofuranosyl 1-pyrophosphate; RNase, ribonuclease; $NaDodSO_4$, sodium dodecyl sulfate.

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reaction mixture at various times by piercing the serum vial septum with a Hamilton syringe and immediately assayed for amidotransferase activity. Eight to ten time points could be taken regularly without altering the atmosphere of the sealed vial. Activities from such points were plotted on semilog paper, and half-lives were determined graphically. The reaction mixture was never flushed with O_2 after addition of enzyme, because bubbling with O_2 or argon resulted in total loss of activity in less than 2 min.

The stability of the nucleotides during preliminary flushing with O_2 and during the inactivation was checked by spotting samples on poly(ethylenimine) sheets and developing the nucleotides in 0.3 M KP_i, pH 7.0. Nucleotides were visualized with a UV lamp and compared to a control solution. No breakdown of nucleotides was observed.

Commercial preparations of GDP and ADP were further purified to remove monophosphates by passage over a DEAE-cellulose column equilibrated in 0.05 M NH_4HCO_3, pH 7.5, buffer. Elution was accomplished with a 0.05–0.5 M NH_4HCO_3, pH 7.5, gradient. The center fraction of the major UV-absorbing component was collected and lyophilized twice to remove the NH_4HCO_3 buffer. The dry powder was dissolved in 50 mM Tris-HCl, pH 7.9, frozen with liquid nitrogen, and stored at −70 °C. This material was judged by chromatography on poly(ethylenimine) sheets to be more pure than commercial preparations.

N-[ethyl-^{14}C]Ethylmaleimide Labeling of the Amidotransferase. Radiochemical labeling of the amidotransferase was accomplished by modification with [^{14}C]NEM and comparing the extent of the reaction with [^{14}C]NEM labeling of ribonuclease A. A stock solution of [^{14}C]NEM was obtained by adding 50 μL of commercial [^{14}C]NEM in pentane to 950 μL of unlabeled 10 mM NEM in Tris-HCl, pH 7.9. Native amidotransferase was labeled by incubation of 200 μL of enzyme (about 1–2 mg) with 600 μL of Tris-HCl, pH 7.9, and 200 μL of [^{14}C]NEM. Denatured enzyme was labeled by incubating 200 μL of enzyme plus 600 μL of Tris-HCl, pH 7.9, containing 5% $NaDodSO_4$ for 15 min prior to [^{14}C]NEM addition. Enzyme that was both denatured and reduced was labeled by incubation of 200 μL of enzyme plus 600 μL of Tris-HCl, pH 7.9, containing 5% $NaDodSO_4$ for 15 min followed by addition of solid $NaBH_4$ (5 mM final concentration) for 15 min prior to addition of [^{14}C]NEM. Modification was allowed to proceed for 60 min with the native enzyme and for 15 min with the denatured and the denatured and reduced enzyme. In all cases, the reaction was stopped by addition of 2 μL of 2-mercaptoethanol. The reaction mixtures were dialyzed extensively to remove all reactants and unreacted [^{14}C]NEM. The concentration of the samples was determined spectrophotometrically, and 0.1-mL enzyme samples were counted in a scintillation fluid that contained 25% Triton X-114, 75% xylene, and 0.4% 2,5-diphenyloxazole. In all cases, the equivalent of 20000–50000 cpm was incorporated per mg of enzyme.

Quantitation of the number of moles of [^{14}C]NEM reacted per subunit of enzyme was achieved by comparison to the [^{14}C]NEM labeling of the eight sulfhydryl groups of reduced RNase A. Modification of 1–2 mg of reduced RNase A was conducted exactly as for modification of native amidotransferase described above. Reduced RNase A was generated by the method of White (1972). Reduced RNase A concentrations were determined by using the extinction coefficient of 8.55×10^3 M^{-1} cm^{-1} at 280 nm (White, 1972). Estimation of the extent of reduction of RNase A was made by spectrophotometric titration with PCMB; 8.0 ± 0.1 free sulfhydryl groups per molecule were routinely observed by using this method.

Sulfhydryl Titrations. Titrations of the enzyme with DTNB were conducted as described by Ellman (1959). The number of moles of DTNB reacted per subunit was determined by monitoring the change in the absorbance at 412 by using an extinction coefficient of 13.6 mM^{-1} cm^{-1}. For titration of the amidotransferase under denaturing conditions, the enzyme was allowed to incubate 15 min with denaturant prior to DTNB addition.

Titration of the amidotransferase with PCMB was performed according to Swenson & Boyer (1957). Quantitation was based upon the change in the extinction coefficient of 7600 M^{-1} cm^{-1} at 250 nm. Native amidotransferase precipitated when it was modified with PCMB at room temperature, but PCMB titrations of native enzyme could be carried out at 4 °C. Denatured enzyme was obtained as described for DTNB titrations.

Silver chloride activity titrations were performed by incubation of native enzyme with increasing quantities of AgCl for 5 min prior to enzymatic assay. The number of moles of Ag^+ reacting per mole of subunit was estimated graphically by extrapolation to zero enzymatic activity.

Determination of Enzyme-Bound S^0. Sulfur in the oxidation state of zero was determined as thiocyanate by using the assay of Sörbo (1957) as modified by Petering et al. (1971). For good estimation of S^0, it was crucial to remove all iron by chelation with 1,10-phenanthroline. Apoprotein precipitated from solution during such treatment. Estimation of the protein content of such mixtures was made by rapid suspension with a Vortex mixer and dissolving a small aliquot of the mixture in the 0.4% NaOH used in the Lowry protein assay (Lowry et al., 1951).

Preparation of Mössbauer Samples. Samples of ^{57}Fe-substituted amidotransferase used for Mössbauer analysis were prepared and analyzed as previously described (Averill et al., 1980), but the native enzyme was inactivated by deliberate exposure to O_2. The buffer used contained 50 mM Tris-HCl, pH 7.9, but no EDTA or other chelating agent.

Results

Chemical Changes Accompanying Reaction of Amidotransferase with Oxygen. We have previously reported that reaction of amidotransferase with O_2 results in partial bleaching of the iron–sulfur chromophore (60% decrease in absorbance at 400 nm), conversion of the inorganic sulfide to a form no longer assayable as S^{2-}, and oxidation of iron in the enzyme to a form, presumably Fe^{3+}, that was assayable with Fe^{2+} chelators only after reduction (Switzer et al., 1979a; Wong, 1978). The Mössbauer spectrum of the ^{57}Fe-substituted enzyme after complete inactivation by exposure to O_2 is indicative of high-spin Fe^{3+} in a rather heterogeneous environment (Figure 1). The spectrum is quite similar to that reported for oxidized nitrogenase by Smith & Lang (1974). Gel filtration of the inactivated enzyme on Sephadex G-25, followed by analysis of the fractions for iron by the method of Fischer & Price (1964), showed that all of the iron remains bound to the protein after oxidation.

Petering et al. (1971) reported that a major portion of the S^{2-} content of ferredoxins is converted to S^0 in thiocystine residues during oxidation. We tested for formation of thiocystine in a 20-mg sample of O_2-oxidized amidotransferase by treatment with CN^- to form SCN^-, which is determined colorimetrically with Fe^{3+} (Sörbo, 1957; Petering et al., 1971). Approximately 0.9 mol of S^0 per mol of subunit was detected. Since this enzyme preparation contained about 3 atoms of S^{2-}

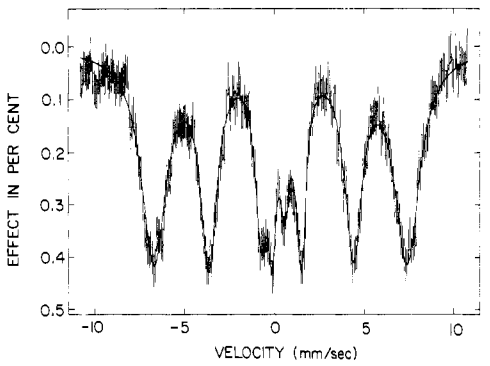

FIGURE 1: Mössbauer spectrum of amidotransferase after complete inactivation by exposure to O_2. The sample of ^{57}Fe-substituted amidotransferase (20 mg/mL) has less than 3% of the native activity and was frozen as a suspension of precipitated protein. The spectrum was taken at 1.8 K in a magnetic field of 440 G perpendicular to the γ-ray beam.

Table I: Reaction of Native and Denatured Amidotransferase with Sulfhydryl and S^{2-} Reactive Agents

sample analyzed	method of analysis	groups reacted per subunit	no. of determinations
native amidotransferase	DTNB	3.0 ± 0.2	2
native amidotransferase	[^{14}C]NEM	3.0 ± 0.3	4
native amidotransferase	AgCl activity titration	16	1
native amidotransferase	PCMB	16 ± 0.1	3
amidotransferase in 1% $NaDodSO_4$ and O_2 for 15 min	DTNB	5.0 ± 0.25	15
amidotransferase in 1% $NaDodSO_4$ and O_2 for 15 min	PCMB	5.3 ± 0.2	2
amidotransferase in 1% $NaDodSO_4$ and O_2 for 15 min	[^{14}C]NEM	5.05 ± 0.15	2
amidotransferase in 1% $NaDodSO_4$ and O_2 for 15 min followed by reduction with $NaBH_4$	[^{14}C]NEM	7.1 ± 0.1	3

per subunit and ≤10% of the initial S^{2-} remained after oxidation (Switzer et al., 1979a), about 2 atoms of S^{2-} per subunit were converted to other unidentified products, possibly SO_3^{2-} or SO_4^{2-}.

Amidotransferase contains seven half-cystine residues per subunit (Wong et al., 1981). From an extensive series of analyses of amidotransferase with various sulfhydryl-reactive reagents (Table I), the following facts emerged. Three sulfhydryl groups per subunit were reactive in the native enzyme. After reaction of these three groups, the enzyme was catalytically inactive (data not shown). The remaining four half-cystine residues were apparently present as cysteinyl groups ligated to the 4Fe–4S center of the enzyme. Titration with reagents that disrupt this center yielded about 16 reactive groups per subunit [expected: 4 S^{2-} at 2 equiv per S^{2-} plus 7 cysteinyl residues for a total of 15 reactive groups (Malkin & Rabinowitz, 1967)]. Seven cysteinyl residues were also detected by reaction with [^{14}C]NEM after denaturation in $NaDodSO_4$, followed by reduction with $NaBH_4$. It was not possible to determine the number of sulfhydryl groups of amidotransferase that had been oxidized in the usual manner, because the oxidized enzyme precipitates at the concentrations needed for analysis. Analysis of such precipitated protein after dissolution in 5% $NaDodSO_4$ indicated less than 0.5 free sulfhydryl group per subunit in a PCMB titration. Analysis of the soluble portion of the oxidized enzyme by PCMB titration yielded 5.2 sulfhydryl groups per subunit. Thus, aggregation appears to be associated with formation of disulfide bonds. When native amidotransferase was dissolved in 1% $NaDodSO_4$ and allowed to react with air for 15 min at room temperature, five sulfhydryl groups were consistently found (Table I). Thus, under these conditions, two cysteinyl residues become unreactive, probably because of formation of a cystinyl or a thiocystinyl group by oxidation.

The amino acid composition of air-oxidized amidotransferase after acid hydrolysis was essentially identical with that of the native enzyme, indicating absence of reaction at other residues. (No attempt to obtain quantitative analyses for half-cystine or tryptophan was made in this determination.) The apparent molecular weight of the amidotransferase subunit on $NaDodSO_4$–polyacrylamide gel electrophoresis was identical with that of the native enzyme, indicating absence of endoproteolytic cleavage of the enzyme during inactivation.

Nature of the Species Involved in Reaction with Amidotransferase. Although no exogenous reductant was added during oxidative inactivation of amidotransferase, it is possible that a reactive oxygen species, such as superoxide, peroxide, hydroxyl radical, or singlet oxygen, is intermediate in the reaction. To test these possibilities, the rate of inactivation of the enzyme by O_2-saturated buffer under standard conditions was tested in the presence of the following traps for reactive oxygen species: 100 mM mannitol, 100 mM methanol, 20 mM thiourea, 20 mM sodium benzoate, 20 mM methional, 5 mM L-histidine, 20 mM 1,4-diazabicyclo-[2.2.2]octane, 5 μg/mL superoxide dismutase, and 10 μg/mL catalase. In all cases, except with thiourea and methional, the half-lives of amidotransferase activity were the same (26 ± 3 min) as that of the control sample. Thiourea and methional caused increases in the rate of inactivation, giving half-lives of 12 and 8 min, respectively. The mechanism of this acceleration is obscure, but the failure of the various traps to inhibit inactivation argues against the participation of these various reactive oxygen species as free intermediates. The inactivation also was shown to proceed at a normal rate in the dark.

Changes in the Physical Properties of Amidotransferase upon Oxidation. In addition to the partial bleaching of the visible spectrum (Wong et al., 1977), amidotransferase underwent other dramatic changes in physical properties during oxidative inactivation. The enzyme became much less soluble and gradually precipitated from solution. A saturated solution of oxidized enzyme in 50 mM Tris-HCl, pH 7.9, at 25 °C contained about 0.8 mg of protein/mL, whereas the native enzyme was soluble at all concentrations tested up to 25 mg/mL. Precipitation occurred more slowly than the loss of enzymatic activity. In the presence of 10 mM Mg^{2+}, the oxidized protein precipitated much more rapidly; Mg^{2+} ions had no effect on the solubility of the native enzyme. Even the soluble portion of the oxidized enzyme was highly aggregated, judging from the fact that the protein was excluded from an Ultrogel AcA-34 gel filtration column.

Further evidence for a major change in the conformation of the amidotransferase protein upon oxidation was obtained from comparison of the CD spectra of native and oxidized amidotransferase (Figure 2). The dichroism of the iron–sulfur chromophore was almost completely abolished upon oxidation, as expected (Figure 2B). Furthermore, the CD spectrum of oxidized amidotransferase in the far-ultraviolet region, which reflects polypeptide folding, was quite different from that for the native enzyme (Figure 2A). Qualitatively, these changes

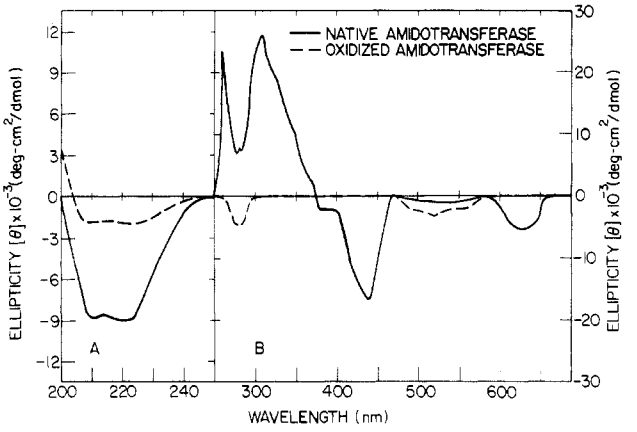

FIGURE 2: CD spectra of native (—) and oxidized (- - -) amidotransferase. Spectra were obtained with a Jasco 40A recording spectropolarimeter at ambient temperature with 0.3–0.6 mg of protein/mL in 50 mM Tris-HCl, pH 7.9. (A) Path length of 1 mm; ellipticity is given in deg·cm^2/dmol of amino acid residues, calculated from the mean residue weight; (B) 1-cm path length; ellipticity is given in deg·cm^2/dmol of the iron–sulfur cluster.

correspond to a complete loss of α-helical conformation during inactivation (Greenfield & Fasman, 1969).

Experimental Variables Influencing the Rate of Inactivation of Amidotransferase by Oxygen. Under standard conditions (50 mM Tris-HCl, pH 7.9, 10 mM Mg^{2+}, and 2.5 mM EGTA, 37 °C, buffer saturated with O_2), amidotransferase activity was lost with a half-life of 26 ± 3 min. This rate as independent of amidotransferase concentration within the range examined (10–300 μg/mL). When the buffer was kept anaerobic by saturating with argon, amidotransferase activity was completely stable at 37 °C for at least 3 h. The half-life was increased 5-fold (to 135 min) when the buffer was saturated with air instead of O_2. These observations, while incomplete, indicate that the reaction is pseudo first order in pO_2.

Within the range from 25 to 41 °C, the rate of inactivation increased slowly with temperature, corresponding to an Arrhenius activation energy of 4.0 kcal/mol. The temperature dependence deviated sharply from this relation at 4 °C, however, where the enzyme was much more stable than would have been predicted. The half-life at 4 °C under O_2 was 280 min.

The rate of inactivation was sharply dependent on pH and the presence of EGTA, which presumably acts by chelating trace metals in the buffer or enzyme preparation (Figure 3). For this reason, EGTA was routinely included in all inactivation mixtures. When various metal ions (Fe^{3+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, and Mn^{2+}, in order of decreasing effectiveness) were added to inactivation mixtures, the half-life of amidotransferase was decreased from 2- to 5-fold. The kinetics of inactivation were not always first order, however, and it is possible that some of these metals catalyzed reactions of sulfhydryl groups, as well as participating in the oxidation of the iron–sulfur center. Interestingly, Mg^{2+}, which is required by amidotransferase for both activity and sensitivity to allosteric effectors (Meyer & Switzer, 1979), did not alter the rate of inactivation of amidotransferase at any concentration up to 15 mM.

Thiol compounds such as dithiothreitol, dithioerythritol, reduced glutathione, and 2-mercaptoethanol at 5 mM decreased the half-life of amidotransferase under O_2 from 26 to 16–21 min; i.e., thiols did not protect the enzyme from oxidative inactivation. This is in direct contrast to other oxygen-sensitive, iron-containing enzymes, which are protected by thiols against oxygen inactivation [e.g., see Kuhn et al. (1980)].

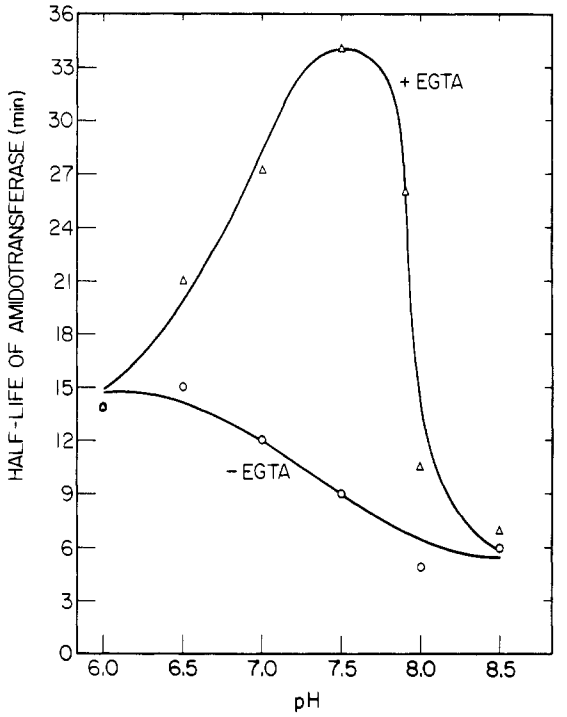

FIGURE 3: Dependence of the oxidative inactivation of amidotransferase on the pH and chelators. Standard reaction conditions were used, except that $MgCl_2$ was omitted and the pH was as shown. (○) No EGTA was added; (△) 2.5 mM EGTA was added.

Table II: Effects of Substrates and Products on the Rate of Inactivation of Amidotransferase by Oxygen

addition(s) to standard inactivation mixture	half-life of amidotransferase (min)
none	26 ± 3
2 mM P-Rib-PP	21
9 mM P-Rib-PP	20
8 mM P-Rib-PP (Mg^{2+} omitted)	15
12 mM glutamine	25
5 mM glutamate	32
22 mM[a] NH_4^+ + 1 mM Rib-5-P	32
5 mM glutamate + 22 mM[a] NH_4^+ + 1 mM Rib-5-P	33
5 mM K-PP_i + 5 mM glutamate + 5 mM Rib-5-P + 109 mM[b] NH_4^+	26

[a] Total ammonium concentration; at pH 7.9, the free NH_3 concentration is 1 mM. [b] Total ammonium concentration; at pH 7.9, the free NH_3 concentration is 5 mM.

Modulation of the Inactivation of Amidotransferase by Substrates and Allosteric Effectors. On the basis of preliminary studies in crude extracts, Turnbough & Switzer (1975b) proposed that the stability of amidotransferase in vivo might be regulated by its ligands. We report here the results of detailed studies of the effects of such metabolites on the rate of inactivation of purified amidotransferase under standardized conditions.

The substrates and products of amidotransferase had relatively little effect on the rate of inactivation (Table II). P-Rib-PP destabilized the enzyme somewhat. Glutamate and a mixture of ribose-5-P and NH_3 (added in place of the product phosphoribosylamine) stabilized the enzyme slightly. An estimate of the effect of both substrates together during catalysis was obtained by analysis of the rate of glutamate production from 20 mM P-Rib-PP plus 20 mM glutamine and 20 mM Mg^{2+} in O_2-saturated buffer, as compared to an anaerobic sample. The difference in the rates of product formation indicated a first-order decay of amidotransferase

Table III: Effects of Nucleotides on the Rate of Inactivation of Amidotransferase by Reaction with Oxygen

inhibitor	effect on inactivation at saturation[a]	concn giving half-maximal effect (mM)	shape of concn dependence curve	effect of 5 mM P-Rib-PP
AMP	stabilizes to 170 min	0.15	complex, sigmoid	antagonizes stabilization
GMP	destabilizes to 12 min	0.26	sigmoid	enhances destabilization
GDP	destabilizes to 16 min	0.6	sigmoid	antagonizes destabilization
ADP	none		none	
ADP + GMP (equimolar)	destabilizes to 8 min	0.05	hyperbolic	antagonizes destabilization
ADP + GDP (equimolar)	destabilizes to 6 min	0.2	sigmoid	none
GMP + GDP (equimolar)	destabilizes to 12 min	0.3	sigmoid (hyperbolic with P-Rib-PP)	enhances destabilization

[a] Times given are half-lives at saturating concentrations of effector.

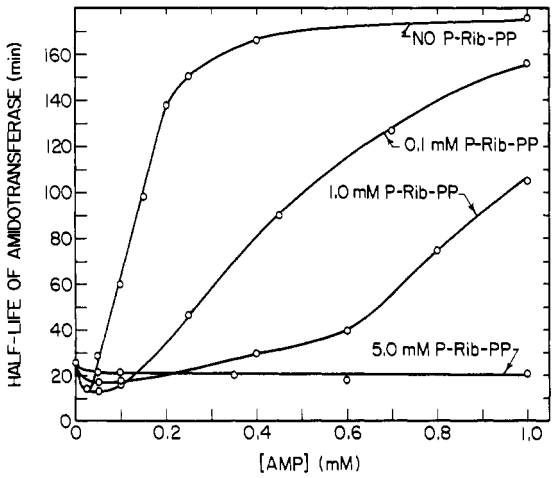

FIGURE 4: Modulation of oxidative inactivation of amidotransferase by AMP and P-Rib-PP. Standard reaction conditions were used, except that the concentrations shown for AMP and P-Rib-PP were added.

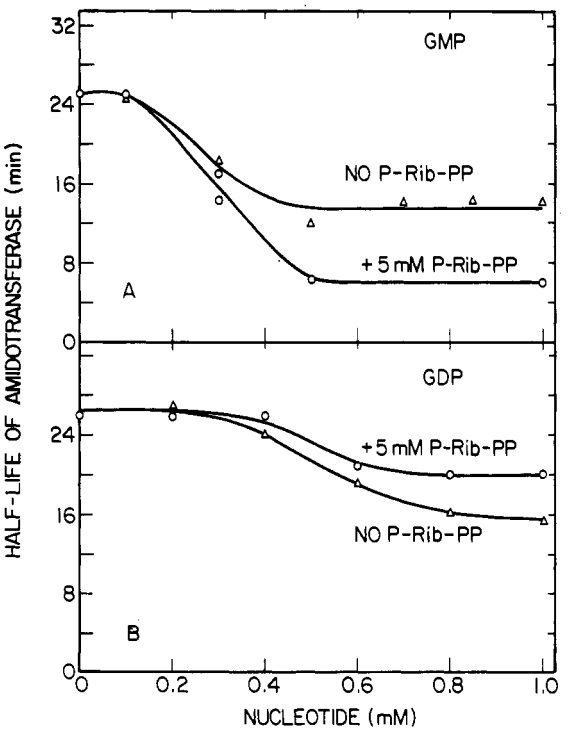

FIGURE 5: Modulation of oxidative inactivation of amidotransferase by GMP (A) and GDP (B). Standard reaction conditions were used without (▲) and the (○) 5 mM P-Rib-PP and the concentrations of nucleotides shown.

with a half-life of 42 min, implying a modest stabilization of the enzyme by both substrates during catalysis.

A survey of a large number of nucleotides and other metabolites including cyclic nucleotides, nucleotide analogues,

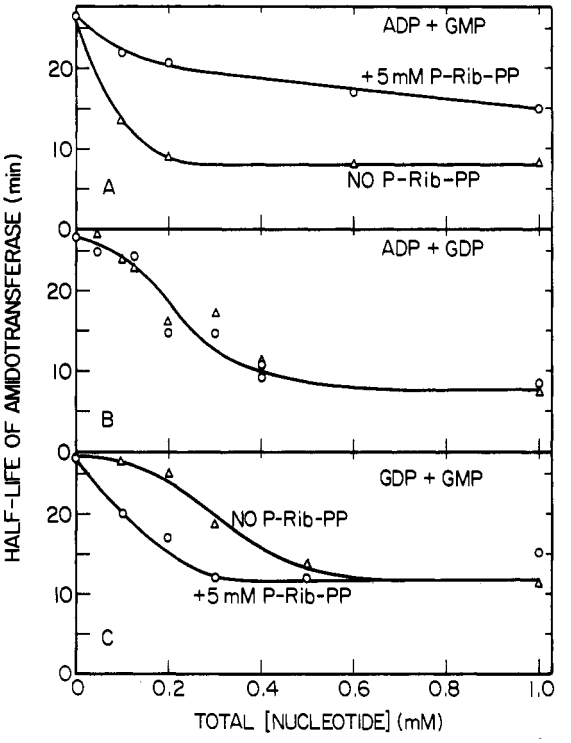

FIGURE 6: Modulation of oxidative inactivation of amidotransferase by pairs of synergistically inhibitory nucleotides. Standard reaction conditions were used without (▲) and the (○) 5 mM P-Rib-PP. (A) Equimolar ADP and GMP; (B) equimolar ADP and GDP; (C) equimolar GDP and GMP were added at the concentrations shown.

and amino acids showed that only three purine nucleotides—AMP, GMP, and GDP—had significant effects on the rate of inactivation of amidotransferase at 1 mM concentrations (Table III). These nucleotides are also the most effective allosteric inhibitors of the enzyme (Meyer & Switzer, 1979). The only other effective allosteric inhibitor, ADP, had little effect on the inactivation by itself. The following points require emphasis.

(1) AMP was the only stabilizing metabolite, yielding a 6–7-fold increase in half-life. The effects of AMP as a stabilizer required Mg^{2+} and were strongly antagonized by the substrate P-Rib-PP (Figure 4), as was the case for allosteric inhibition (Meyer & Switzer, 1979). The effects of AMP were highly specific; no structural analogue was found that gave any stabilization (data not shown).

(2) GMP and GDP were effective destabilizers of amidotransferase to inactivation by O_2 (Figure 5). Curiously, P-Rib-PP antagonized destabilization by GDP but enhanced destabilization by GMP.

(3) All three pairs of nucleotides previously shown to be synergistic inhibitors of amidotransferase (Meyer & Switzer, 1979) were very effective destabilizers (Figure 6). ADP, which had little effect by itself, was a partner in two of these

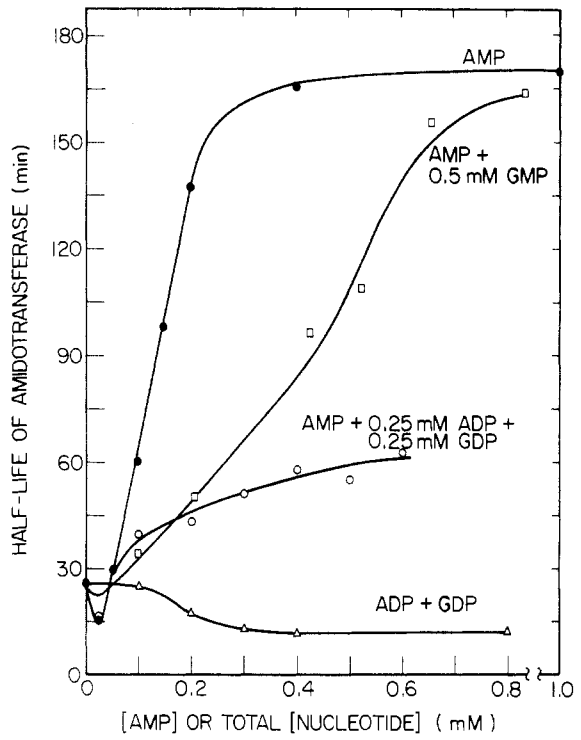

FIGURE 7: Competition between stabilization of amidotransferase by AMP and destabilization by ADP plus GDP to oxidative inactivation. Standard reaction conditions were used with increasing concentrations of nucleotides added as follows: (●) AMP; (△) equimolar ADP and GDP; (□) AMP plus 0.5 mM GMP at all AMP concentration levels; (○) AMP plus 0.25 mM ADP and 0.25 mM GDP at all AMP concentration levels.

pairs. The effect of P-Rib-PP on the action of each synergistic pair was different.

(4) There was an antagonistic relationship between the effects of the stabilizer AMP and destabilizing nucleotides, whether acting singly (as with GMP) or as synergistic pairs (as with ADP + GDP) (Figure 7). A sufficient excess of either effector was able to overcome the effects of the other completely, but the half-life of amidotransferase was sensitive to the absolute concentrations of effectors as well as the ratio of their concentrations (data not shown). At low concentrations of AMP, P-Rib-PP destabilized in such mixtures, but at higher concentrations of AMP, it had little effect.

Discussion

Chemistry of Inactivation of Amidotransferase. The chemical events accompanying the inactivation of amidotransferase by O_2 may be summarized as follows (Figure 8): The iron in the iron–sulfur cluster is oxidized to the high-spin ferric state, apparently bound in a heterogeneous fashion to the protein. About one-third of the sulfide is oxidized to S^0 in thiocystine residues, while the remainder is oxidized to unidentified products. This is in contrast to a number of ferredoxins, in which 50–80% of the sulfide was oxidized to S^0 (Petering et al., 1971). The limited solubility of oxidized amidotransferase hindered direct studies of the fate of the sulfhydryl groups of the enzyme during inactivation, but the following picture fits all of our observations. We suggest that two of the four cysteine residues that are ligated to the iron–sulfur cluster are converted to a thiocystine residue and that the other two remain, at least initially, as free cysteinyl groups. Consistent with this picture is the observation that NEM-treated native amidotransferase had two sulfhydryl groups remaining titratable by PCMB after it was completely oxidized. Similarly, when amidotransferase was oxidized in the

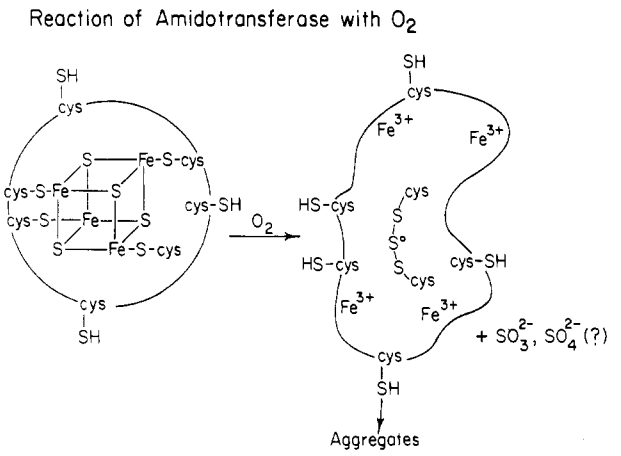

FIGURE 8: Schematic summary of the reaction of amidotransferase with oxygen.

presence of $NaDodSO_4$, five sulfhydryl groups remained (three groups that were not ligated to the iron–sulfur cluster plus two of the four that were ligated to the cluster). Ultimately, however, most of the five sulfhydryl groups of the oxidized enzyme are probably oxidized to disulfides in the aggregated oxidized enzyme.

Implications Concerning the Allosteric Nature of Amidotransferase. The results of studies in the preceding paper (Wong et al., 1981) showed that whereas the allosteric inhibitors of amidotransferase have effects on the distribution of the enzyme among monomeric, dimeric, and tetrameric states, there is no correlation between inhibition and quaternary structure. It is also clear that susceptibility of amidotransferase to oxidative inactivation is not directly correlated with quaternary structure or with a single "inhibited conformation". For example, both AMP, a stabilizer, and GMP, a destabilizer, promote dimer formation, while GDP alone and in synergistic pairs is a destabilizer and promotes tetramer formation. All three nucleotides are allosteric inhibitors.

There is also a substantial difference between the concentrations of nucleotides needed to exert maximal effects on reactivity with oxygen and the concentrations needed to inhibit amidotransferase activity, especially with the destabilizing nucleotides. In general, the concentration of the destabilizing nucleotides or synergistic pairs needed to give half-maximal destabilization (Table III) is 10–40-fold lower than the concentration needed to give 50% inhibition (Meyer & Switzer, 1979). This is true whether or not P-Rib-PP is present in the inactivation mixture. In the case of AMP stabilization and inhibition, however, the concentration dependencies of the effects and their reversal by P-Rib-PP are similar, which suggests a possible correlation between the "inhibited" and "stabilized" conformations in this case only. The observation that amidotransferase is destabilized and stabilized at nucleotide concentrations far below those needed to inhibit the enzyme indicates that the nucleotides can act to regulate the stability of a catalytically active—as opposed to a fully inhibited—enzyme. This is to be expected if regulation of oxidative inactivation is a physiologically important phenomenon.

Meyer & Switzer (1979) concluded that AMP binds to a highly specific allosteric site on *B. subtilis* amidotransferase. The data in this paper reinforce that conclusion. AMP was the only ligand to stabilize amidotransferase against O_2; a large number of AMP analogues either were without effect or were weak destabilizers (data not shown). The effects of destabilizing inhibitory nucleotides must be mediated via binding at another allosteric site. In fact, the specificity and capacity for synergism of these destabilizing nucleotides strongly suggest

the existence of two additional nucleotide binding sites per subunit. Altogether, the properties of amidotransferase are impossible to reconcile with a two-state model for allosteric behavior (Monod et al., 1965) and suggest that the enzyme possesses extraordinary conformational flexibility.

Implications Concerning the Regulation of Amidotransferase Inactivation and Degradation in Vivo. On the basis of experiments with *B. subtilis* cultures, Turnbough & Switzer (1975b) demonstrated that the inactivation of amidotransferase in vivo specifically requires O_2 and that stability of the enzyme is controlled by the culture conditions. If the stability of amidotransferase to O_2 in vivo is regulated by ligands of the enzyme as proposed by Turnbough & Switzer (1975a), the results of this paper suggest the following as the simplest model for these effects. In growing cells, in which amidotransferase is stable (Turnbough & Switzer, 1975a), the total concentration of destabilizing nucleotides (primarily GMP, GDP, and ADP) is quite low, and the enzyme is probably stabilized significantly by AMP. In starving cells or other conditions in which the enzyme is unstable, we propose that the total concentration of destabilizing nucleotides rises severalfold. The AMP concentration may also rise under these conditions, but the net effect would be destabilization of amidotransferase (e.g., in the presence of 1.0 mM each of AMP, ADP, GMP, and GDP, the half-life of the enzyme is 15 min; this is equivalent to a half-life under air of about 75 min, which is close to the half-life observed in vivo). As a first approximation, we suggest that the effects of P-Rib-PP concentration are secondary, because the P-Rib-PP concentration probably never exceeds 400 μM (Sadler & Switzer, 1977). Obviously, the validity of this model is unproven. We are currently attempting to test the model by determining whether the stability of amidotransferase and the size of metabolite pools behave in the predicted manner under a variety of culture conditions.

We have shown that all protein that is immunochemically cross-reactive with either native or oxidized amidotransferase disappears from stationary *B. subtilis* cells (Switzer et al., 1979a,b). This suggests that the enzyme is degraded. We propose that the oxidized enzyme described here is also formed in vivo and that it is the true target for proteolysis. Certainly, the marked changes in protein structure that accompany oxidation would be expected to make the protein more susceptible to proteolysis, as is usual for denatured proteins (Goldberg & Dice, 1974).

Acknowledgments

We gratefully acknowledge Drs. P. Debrunner, A. Dwivedi, and J. Y. Wong for preparation of samples and determination of Mössbauer spectra and Simon Rosenzweig for mass culturing bacteria. Dr. J. Y. Wong conducted preliminary experiments on some aspects of the work reported here.

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