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Purification and Properties of Glutamine Phosphoribosylpyrophosphate Amidotransferase from *Bacillus subtilis*†

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ABSTRACT: A procedure for the rapid and efficient purification of glutamine phosphoribosylpyrophosphate amidotransferase to better than 98% homogeneity from derepressed *Bacillus subtilis* cells is described. The molecular weight of the subunit was estimated to be about 50 000. The purified enzyme exhibits microheterogeneity on electrophoresis on highly resolving polyacrylamide gels; it is suggested that this heterogeneity results from limited proteolytic modification of the native subunit. The native enzyme exists in equilibrium among tetrameric, dimeric, and monomeric forms. The influence of enzyme concentration and the presence of substrates and allosteric inhibitors on this equilibrium are described. There is no simple correlation between allosteric inhibition and stabilization of dimeric or tetrameric states. The amino acid composition of the amidotransferase is reported; presence of a 4Fe–4S center in the enzyme was described previously. Preparation of inactive apoprotein by treatment with 1,10-phenanthroline and general characteristics of the apoprotein are presented.

Glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14), hereafter called "amidotransferase", catalyzes the first reaction of purine nucleotide biosynthesis de novo and has received considerable attention as a major site of regulation of this pathway (Wyngaarden, 1972, 1973). Our attention to the amidotransferase of *Bacillus subtilis* grew out of the observation that the enzyme is inactivated in stationary cells in a reaction that requires O_2 (Turnbough & Switzer, 1975a). The enzyme is also oxygen labile in vitro (Turnbough & Switzer, 1975b). The site of reaction with oxygen in vitro was found to be a 4Fe–4S cluster covalently bound to the enzyme (Wong et al., 1977; Averill et al., 1980). The enzyme has thus become an object of considerable interest, from the perspective both of novel regulatory mechanisms and of novel functions

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for iron–sulfur centers in enzymes. This paper presents details of a rapid, efficient procedure for purifying *B. subtilis* amidotransferase, for which no purification procedure has been previously reported, and a general physical and chemical characterization of the purified enzyme.

Experimental Procedures

Growth of Bacterial Cells. Amidotransferase was isolated from purine-starved cells of *B. subtilis* strain 60164 (*purB*, Pur^-, Thi^-; kindly provided by Dr. Ernst Freese, NIH, Bethesda, MD), which was grown at 37 °C in a 200-L New Brunswick fermentor with vigorous aeration (6 ft^3/min). The growth medium contained per liter the following: 1.5 g of yeast extract (Gibco), 2.27 g of Tryptose (Difco), 5 g of glucose, 3.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 5 mg of $MnCl_2 \cdot 4H_2O$, and 12.5 mg of adenine dihydrate. Cells were harvested by centrifugation with a Sharples centrifuge 1 h after the depletion of purines from the medium, which was evident from a sharp decrease in growth rate. Harvesting required 45–60 min; waiting longer to harvest often resulted in extensive inactivation of amidotransferase. The cell paste (about 1 kg wet weight) was washed by suspending very briefly in a Waring blender with an equal volume of cold 50 mM Tris-HCl buffer, pH 7.9, containing 1 M KCl and 1.0 mM phenylmethanesulfonyl fluoride. The suspended cells were centrifuged at 25000*g* for 5 min, and the precipitated cells were suspended as described above in cold Tris-HCl, pH 7.9, containing 1.0 mM phenylmethanesulfonyl fluoride. The cells were collected by centrifuging as described above, frozen in liquid nitrogen, and stored at −70 °C. The total time to wash the cells was about 45 min with a recovery of 80–90% by weight.

Assays. Amidotransferase activity was assayed spectrophotometrically as described by Meyer & Switzer (1979), except that the concentrations of 5-phospho-α-D-ribofuranosyl 1-pyrophosphate (P-Rib-PP)[1] and $MgCl_2$ were 5.0 and 10 mM, respectively. During purification of amidotransferase, protein was assayed after precipitation with trichloroacetic acid by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The concentration of purified amidotransferase was determined from the absorbance at 278 nm and the extinction coefficient based on dry weight (Wong et al., 1977). Iron and S^{2-} were analyzed as previously described (Averill et al., 1980).

Other Methods. Determination of molecular weights by gel filtration was performed with a 2 × 55 cm column of Sephadex G-200 at 4 °C with a constant pressure head and flow rate. Samples were loaded in 0.5 mL; the buffer was 50 mM Tris-HCl, pH 7.9, at 4 °C, containing 10 mM $MgCl_2$. Molecular weight standards were beef liver catalase, yeast alcohol dehydrogenase, and bovine serum albumin. For protection of amidotransferase from O_2 during chromatography, the buffer reservoir was fitted with a stopper and tube that allow constant flushing of the buffer with argon before and during analysis. Activity was recovered in yields of 80–90%. Constancy of elution volumes of standards was demonstrated throughout the series of analyses.

Sucrose density gradient sedimentation followed the general procedures of Martin & Ames (1961). Linear gradients (10 mL) from 5 to 20% sucrose (Schwarz/Mann, ultrapure grade) in 50 mM Tris-HCl, pH 7.9, containing 10 mM $MgCl_2$ with appropriate additions were prepared. Amidotransferase in 0.25-mL samples with appropriate additions was layered onto the gradients, and the tubes were centrifuged for 16–18 h at 36000 rpm at 4 °C in a Beckman Model L3-50 ultracentrifuge with an SW41 rotor. Catalase was used as an internal standard and was assayed as described by Beers & Sizer (1952). Molecular weights were calculated as described by Martin & Ames (1961), using 250000 for the molecular weight of catalase.

Electrophoresis of native amidotransferase on polyacrylamide tube gels was conducted by using the discontinuous buffer system of Davis (1964). Electrophoresis of enzyme denatured with $NaDodSO_4$ was conducted as described by Weber et al. (1972) for the tube gels and as described by Laemmli (1970) for 20 cm × 20 cm × 1 mm slab gels containing either 10% acrylamide or a 7.5–15% linear gradient in acrylamide.

Amino acid analysis was carried out according to Moore & Stein (1963) with a Beckman Model 121 analyzer. Tryptophan was determined after alkaline hydrolysis according to the procedure of Hugli & Moore (1972).

Materials. P-Rib-PP, glutamate dehydrogenase, acetylpyridine adenine dinucleotide, phenylmethanesulfonyl fluoride, and all nucleotides were purchased from Sigma Chemical Co. L-Glutamine and dithiothreitol were from Calbiochem, 1,10-phenanthroline was from Fischer Scientific Co., sucrose and enzyme-grade $(NH_4)_2SO_4$ were from Schwarz/Mann, electrophoresis-grade acrylamide was from Eastman Kodak Co., bis(acrylamide) was from Bio-Rad, and DEAE-cellulose (DE-52) was from Whatman Ltd. Protamine sulfate was purchased from ICN. Variability from batch to batch in the behavior of protamine sulfate during amidotransferase purification was observed; pilot scale trials are advisable. Buffer A used in amidotransferase purification contained 50 mM Tris-HCl, pH 7.9, at 4 °C, 10 mM $MgCl_2$, and 0.1 mM EDTA.

Results

Purification of Glutamine P-Rib-PP Amidotransferase. All procedures were carried out at 0–4 °C with buffers which had been flushed with argon to remove dissolved oxygen. Where practical, enzyme solutions were kept under a layer of flowing argon gas and loosely covered with Parafilm.

(*A*) *Preparation of Cell Extract.* Frozen cell paste (800–1000 g) was thawed and suspended with a Waring blender in 3 volumes of buffer A containing 2 mM AMP, 10 mM 2-mercaptoethanol, and 1.0 mM phenylmethanesulfonyl fluoride (the latter added from a concentrated ethanol solution). The suspended cells were ruptured by passage 3 times through a Manton-Gaulin mill at 8000 psi. The effluent fluid from each passage was rapidly cooled by immediate passage through an ice-chilled stainless-steel cooling coil ($^3/_8$ in. O.D. by 24 ft long). The suspension of disrupted cells was centrifuged for 1 h at 25000*g*, and the precipitate was discarded. Smaller masses of cells may also be conveniently ruptured by extended sonication with cooling (Wong, 1978).

(*B*) *Protamine Sulfate Precipitation.* A 2.0% (w/v) solution of protamine sulfate in buffer A (containing 2 mM AMP, 5 mM dithiothreitol, and 1.0 mM phenylmethanesulfonyl fluoride and readjusted to pH 7.9 with solid Tris base, if necessary) was added dropwise with stirring over about 30 min to the crude extract to give a final protamine sulfate concentration of 0.4% (w/v). The resulting suspension was stirred with a magnetic stirrer for an additional 45 min under an argon layer. The mixture was then centrifuged for 1 h at 25000*g*, and the precipitate was discarded.

(*C*) *Fractionation on DEAE-cellulose.* The conductivity of the clear yellow supernatant fraction from the protamine

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; P-Rib-PP, 5-phospho-α-D-ribofuranosyl 1-pyrophosphate.

Table I: Purification of Glutamine P-Rib-PP Amidotransferase from *B. subtilis*

fraction	volume (mL)	total activity (units)	protein concn[b] (mg mL^{-1})	sp act. (units mg^{-1})	yield (%)	x-fold purification
crude extract[a]	2600	6140	26.9	0.09	(100)	(1)
protamine sulfate supernatant	3000	5550	11.8	0.16	90	1.8
0.75 M KCl eluate from DEAE-cellulose	1000	4650	1.3	3.6	76	41.8
ammonium sulfate fraction[d]	10	3350	7.7[c]	43.3	55	498

[a] From 1 kg of frozen cell paste. [b] Based on the assay of Lowry et al. (1951) after precipitation of the protein with trichloroacetic acid and washing of the precipitate with ethanol. [c] Based on an absorbance of 278 nm and an extinction coefficient determined from the dry weight (Wong et al., 1977). [d] This preparation contained 3.1 atoms of Fe per subunit.

sulfate treatment was adjusted with solid KCl until it was equivalent to a solution of buffer A containing 2 mM AMP, 5 mM dithiothreitol, and 0.15 M KCl (11.5 mΩ^{-1}, using a Radiometer conductivity meter). The pH was adjusted with solid Tris base to 7.9 if necessary. This solution was stirred with 150 mL of wet packed DE-52 per L of protamine sulfate supernatant fraction for 1 h under argon. The DE-52 was previously equilibrated with buffer A containing 2 mM AMP, 5 mM dithiothreitol, and 0.15 M KCl. The suspension was filtered on a Büchner funnel under mild suction, and the resin was resuspended and washed for 15 min in 1.5–2 L of buffer A containing 2 mM AMP, 5 mM dithiothreitol, and 0.15 M KCl. After being filtered, the bound amidotransferase was eluted from the DE-52 resin by two or three successive washes of 15-min duration with 250 mL per wash of buffer A containing 2 mM AMP, 5 mM dithiothreitol, and 0.75 M KCl. The eluate was collected by gentle vacuum filtration. This DEAE-cellulose fractionation may also be carried out by elution from a column by KCl gradient (Wong, 1978), but the batch procedure is faster and yields enzyme of adequate purity for the next step.

(*D*) *Ammonium Sulfate Fractionation.* Amidotransferase was precipitated from the filtrate eluted from DE-52 in the previous step by dissolving sufficient solid $(NH_4)_2SO_4$ to bring the solution to 40% of saturation (243 g/L). After the solution stood on ice under argon for 1 h, it was centrifuged for 1 h at 25000*g*. The precipitated protein was resuspended in 40 mL of buffer A containing 2 mM AMP, 5 mM dithiothreitol, and 176 g/L $(NH_4)_2SO_4$ (30% of saturation) and centrifuged for 30 min at 25000*g*. The final precipitate was dissolved in 10 mL of buffer A containing 2 mM AMP and 5 mM dithiothreitol, and any insoluble material was removed by centrifuging for 30 min at 25000*g*. The resultant dark brown supernatant solution, which contained highly purified amidotransferase, was frozen and stored in liquid nitrogen. Generally, samples were prepared for subsequent studies by thawing and dialyzing against 50 mM Tris-HCl, pH 7.9, and under argon.

A summary of typical purification is shown in Table I. In general, the enzyme was purified 450–500-fold in 50–60% yield. The procedure described above can be completed in 11 h. The specific activity of the purified enzyme generally varied from 40 to 50 units/mg of protein.

Characterization of Purified Glutamine P-Rib-PP Amidotransferase. (*A*) *Criteria of Purity and Subunit Molecular Weight.* Analysis of purified amidotransferase by electrophoresis in polyacrylamide tube gels containing 0.1% NaDodSO$_4$ and acrylamide monomer concentrations ranging from 5 to 15% indicated the presence of a single major component (Figure 1). Traces of minor contaminants, which were estimated by densitometry of Coomassie Blue stained gel to comprise 2% or less of the total protein, could be detected. These trace contaminants could be removed by repeating the absorption and elution from DEAE-cellulose as described

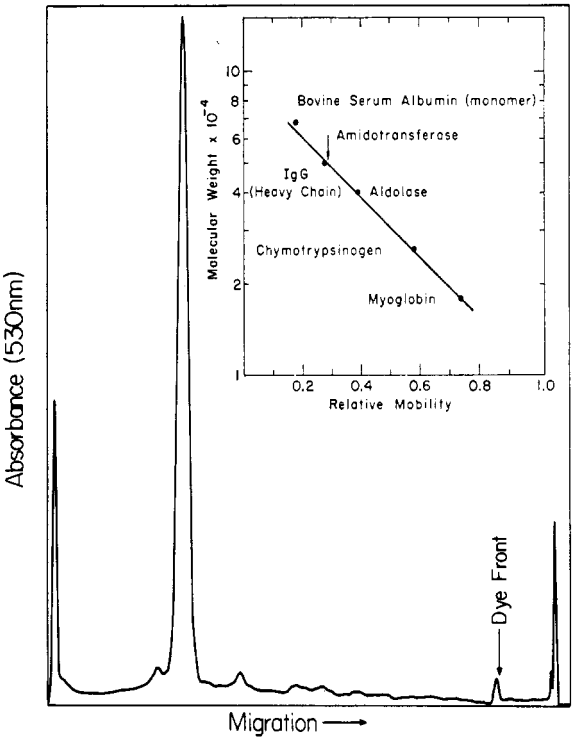

FIGURE 1: Electrophoresis of purified amidotransferase on a 10% polyacrylamide tube gel containing 1% NaDodSO$_4$ (Weber et al., 1972). A 21-μg sample was analyzed, and the gel was stained with Coomassie Blue, destained, and scanned at 530 nm. Inset: Determination of the subunit molecular weight of amidotransferase by comparison to standard proteins.

above. The molecular weight of the amidotransferase subunit was estimated by comparison to standard proteins (Weber et al., 1972) to be 50000 ± 3000 (Figure 1, inset). The presence of a single protein component was also shown by electrophoresis of the native protein by the disk gel procedure of Davis (1964). Evidence based on gel filtration analysis for near homogeneity of the isolated native protein and identity of the major protein with amidotransferase activity has been published previously (Wong et al., 1977).

(*B*) *Evidence for Microheterogeneity.* Although the amidotransferase preparations appeared nearly homogeneous on electrophoresis in NaDodSO$_4$-containing polyacrylamide tube or slab gels, three closely spaced bands of protein could usually be resolved from 20-cm slab gels poured with a gradient from 7.5 to 15% acrylamide (Figure 2).

(*C*) *Effects of Concentration and Ligands on the Molecular Weight of Native Amidotransferase.* When purified amidotransferase was analyzed by gel filtration on a calibrated Sephadex G-200 column, the apparent molecular weight was dependent on the concentration of the sample analyzed (Figure 3A). At high enzyme concentration, the enzyme eluted primarily as a tetramer, although some trailing of lower molecular weight components was generally evident. The average

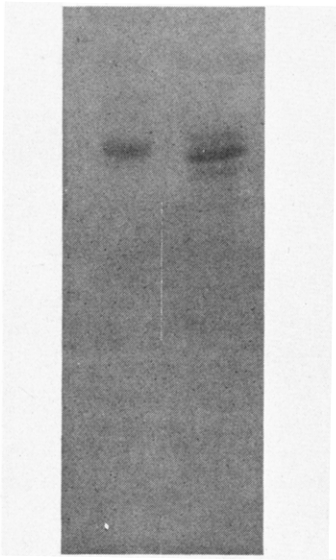

FIGURE 2: Demonstration of microheterogeneity of purified amidotransferase by electrophoresis on $NaDodSO_4$-containing slab gels. (Left) A 1.5-μg sample analyzed on a 10% polyacrylamide slab gel; (right) 2.0 μg analyzed as described for the left panel, except that the slab gel was poured with a 7.5–15% polyacrylamide concentration gradient.

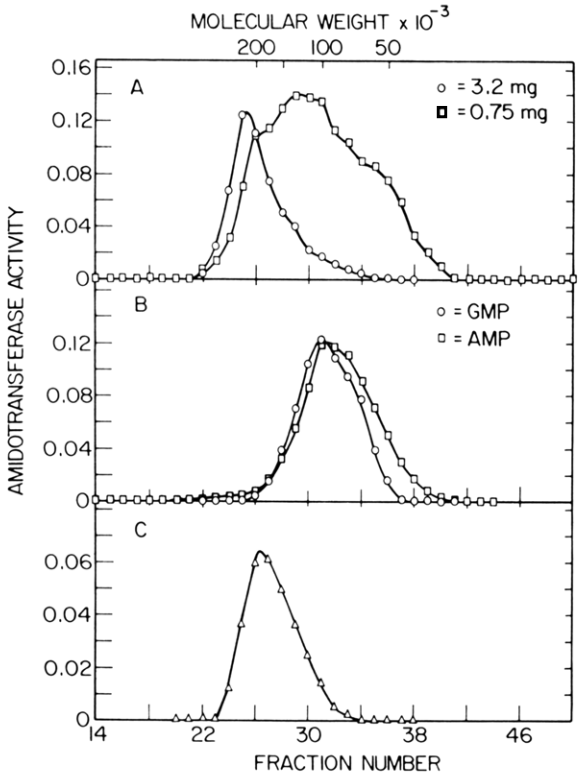

FIGURE 3: Analysis of the state of aggregation of amidotransferase by gel filtration on Sephadex G-200. Conditions of chromatography and standardization with proteins of known molecular weight are given under Experimental Procedures. (A) Dependence on amidotransferase concentration. Samples loaded in 0.5 mL were 3.2 (○) and 0.75 mg (□) of amidotransferase. (B) Effects of 5 mM AMP (□) and 5 mM GMP (○) on the sample (0.75 mg) and chromatography buffers. (C) Effect of 5 mM GDP on the sample (0.75 mg) and chromatography buffers.

of several such determinations gave a molecular weight of 200000 ± 15000. When a lower concentration was examined, the distribution of activity (and protein) indicated multiple states of aggregation, suggestive of an equilibrium among monomeric, dimeric, and tetrameric forms (Figure 3A). The distribution among these forms at low concentrations of the enzyme was markedly affected by allosteric inhibitors. For example, AMP and GMP stabilized the dimeric form (Figure 3B). On the other hand, GDP, which is a weak inhibitor by itself, but an effective partner in synergistic inhibition by certain pairs of nucleotides (Meyer & Switzer, 1979), stabilized the tetrameric form (Figure 3C). Yet a third distribution was observed with 2 mM ADP, a weak inhibitor, or with 2 mM ADP plus 2 mM GMP, an effective synergistic pair. In this case, the activity eluted as a single symmetrical peak with an apparent molecular weight of 150000–160000, suggesting a rapid equilibrium between tetrameric and dimeric forms (data not shown). The effects of nucleotides on the apparent molecular weight of amidotransferase were also dependent on the concentration of the enzyme. For example, at a concentration where the enzyme eluted primarily as a tetramer, 2 mM AMP, which stabilized the dimeric form at low enzyme concentrations, caused the enzyme to elute as though it had a molecular weight of 150000.

Table II: Estimation of the Apparent Molecular Weight of Amidotransferase under Various Conditions by Sedimentation on Sucrose Density Gradients

additions to sample[a]	initial concn of amidotransferase (mg mL^{-1})	app M_r of major component	comments
none	0.02	93000	no minor components
none	0.05	93000	no minor components
none	0.01	93000	no minor components
none	1	93000	no minor components
none	2	110000–125000	double peak
none	4	150000–160000	double peak
none	6.7	185000	small shoulder of lower M_r
none	8	185000	small shoulder of lower M_r
none	10	185000	small shoulder of lower M_r
5 mM P-Rib-PP	0.05	135000	very broad peak
20 mM P-Rib-PP	0.1	135000	very broad peak
20 mM glutamine	0.1	93000	no minor component
5 mM AMP	8	140000	trailing peak
5 mM GMP	8	150000	trailing peak

[a] All samples contained 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, and amidotransferase at the initial concentrations shown.

Most of these generalizations were confirmed by analysis of amidotransferase by sedimentation velocity experiments using sucrose density gradients according to the method of Martin & Ames (1961) (Table II). A conversion of amidotransferase from a dimer to a tetramer within a 10-fold increase in protein concentration was evident. Glutamine had no effect on the apparent molecular weight, whereas P-Rib-PP appeared to shift the equilibrium slightly toward the tetrameric state. AMP and GMP promoted a decrease in the apparent molecular weight, but complete conversion to the dimeric form, as seen on gel filtration, was not obtained.

(*D*) *Amino Acid Composition.* The amino acid composition of *B. subtilis* amidotransferase is shown in Table III. Hydrolyses were conducted in 6 N HCl at 110 °C for 24, 53, 72, and 96 h. The results were averaged and given as the nearest integral value. Serine and threonine analyses were corrected for first-order decay during hydrolysis. The valine and isoleucine values increased during hydrolysis, so the values at 96 h are shown. Seven half-cystine residues per subunit were

Table III: Amino Acid Composition of Glutamine P-Rib-PP Amidotransferase from *B. subtilis*, Pigeon Liver, and *E. coli*

amino acid	residues per subunit		
	B. subtilis	pigeon liver[a]	*E. coli*[b]
cysteic acid	7	18	5
aspartic acid	36	41	66
threonine	27	24	20
serine	34	25	19
glutamic acid	54	34	54
proline	14	24	21
glycine	47	40	38
alanine	34	41	49
valine	35	33	36
methionine	12	9	11
isoleucine	30	20	43
leucine	37	49	40
tyrosine	13	13	17
phenylalanine	12	14	23
lysine	22	28	18
histidine	13	10	10
arginine	20	25	39
tryptophan	1	6	ND[c]

[a] Rowe & Wyngaarden (1968). [b] Messenger & Zalkin (1979). [c] Not determined.

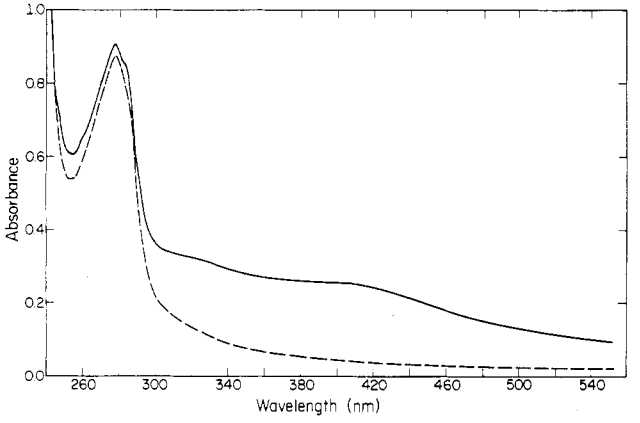

FIGURE 4: Absorption spectra of native amidotransferase (—) and apoprotein prepared by treatment with 1,10-phenanthroline followed by dialysis (---). Both samples were 1.0 mg/mL in 50 mM Tris-HCl, pH 7.9.

found as cysteic acid following hydrolysis in the presence of dimethyl sulfoxide (Spencer & Wold, 1969). In the following paper (Bernlohr & Switzer, 1981), it is shown that all are present as cysteinyl residues, three as sulfhydryl groups and four ligated to an 4Fe–4S center in the enzyme. For comparison, the amino acid compositions of glutamine P-Rib-PP amidotransferases from pigeon liver and *Escherichia coli* are shown; there appears to be little similarity in these compositions.

(*E*) *Properties of the Apoprotein.* The iron–sulfur center of amidotransferase could be gently removed by treatment of the enzyme with a 100-fold molar excess (over iron) of 1,10-phenanthroline under anaerobic conditions at room temperature for 16 h followed by extensive dialysis to remove the Fe^{2+}–phenanthroline complex (Wong et al., 1977). The apoprotein was colorless (Figure 4), contained less than 0.02 atom of Fe or S^{2-} per subunit, and was completely devoid of either the glutamine-dependent of the NH_3-dependent activity. Loss of activity was complete before all of the Fe had reacted (Figure 5). Thus, while the iron–sulfur center is essential for activity, its reaction with 1,10-phenanthroline is probably a complex process in which the individual Fe atoms react at different rates. As isolated after dialysis, the apoprotein was

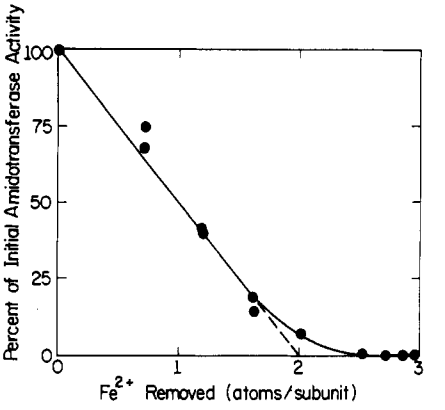

FIGURE 5: Inactivation of amidotransferase by 1,10-phenanthroline. Amidotransferase (1 mg/mL) in 50 mM Tris-HCl, pH 7.9, was incubated with a 50-fold molar excess (over subunit concentration) of 1,10-phenanthroline at room temperature under argon. The extent of removal of Fe^{2+} was determined from the absorption of the Fe^{2+}–(phenanthroline)$_3$ complex at 510 nm (Williams, 1955), and samples were removed for enzyme assay as usual. About 2 h was required under these conditions to remove 50% of the Fe. An equal concentration of 1,7-phenanthroline, which does not chelate Fe^{2+}, gave no inactivation.

highly aggregated; it was excluded from agarose gels with an exclusion limit of 400 000. The apoprotein was much less soluble than the native enzyme and precipitated in the presence of 10 mM Mg^{2+} ions. In these physical properties, the apoprotein resembles the product of inactivating the native protein by reaction of the iron–sulfur center with O_2 (Bernlohr & Switzer, 1981). Analysis of the sulfhydryl group content of the apoprotein by titration with 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959) in 2% $NaDodSO_4$ yielded less than 0.2 sulfhydryl group per subunit.

Discussion

The procedure described here for the purification of amidotransferase from *B. subtilis* cells is rapid, simple, and highly reproducible. Even though amidotransferase is an oxygen-labile enzyme (Bernlohr & Switzer, 1981), protection of the enzyme by inclusion of AMP, exclusion of air, and maintaining temperatures at or below 4 °C, together with the rapidity of the entire procedure, allows isolation of the enzyme in 50–60% yield. The substitution of batchwise absorption and desorption from DEAE-cellulose provided a major saving of time over the column chromatographic procedures used earlier (Wong, 1978) without a significant decrease in the effectiveness of the step. The procedure can be used as described to purify amidotransferase from cells grown on other media, as exemplified by the use of defined low iron medium for the preparation of ^{57}Fe-substituted enzyme for Mössbauer spectroscopy (Averill et al., 1980). It is also possible to substitute other *B. subtilis* strains, such as strain 168 (Trp^-), for the purine auxotroph used routinely. The amidotransferase isolated in such a case has been shown to be indistinguishable from that isolated from strain 60164, but the yield per gram of cell paste was 3–4-fold lower. A procedure for partial purification of *B. subtilis* amidotransferase by affinity chromatography on ATP–agarose is available (Wong & Switzer, 1979) but offers no advantage over the present procedure and would be very expensive for use in large-scale purification.

The biochemical basis of the microheterogeneity of amidotransferase that is observed on electrophoresis of the $NaDodSO_4$-denatured enzyme on polyacrylamide gradient slab gels is not unequivocally established, but we believe it results from limited proteolytic cleavage of the native amidotransferase polypeptide. The following lines of evidence argue

against the idea that our amidotransferase preparations are contaminated with other proteins, whose subunit molecular weights are very nearly the same. (a) The native enzyme appears homogeneous on polyacrylamide disc gel and gel filtration analysis. In the latter case, there is a good correlation between elution of activity and elution of protein even though both can be shifted from one state of aggregation to another. It would be an extraordinary coincidence for contaminating proteins to show these properties. (b) Attempts to remove the microheterogeneity of purified amidotransferase by affinity chromatography on ATP–agarose and by refined $(NH_4)_2SO_4$ fractionation, poly(ethylene glycol) fractionation, hydroxylapatite chromatography, and repeated gradient elution from DEAE-cellulose have all failed to alter the patterns of microheterogeneity of the starting samples, although other trace impurities can be removed.[2] (c) Immunoprecipitation of amidotransferase from cells grown on $^{35}SO_4^{2-}$ followed by radioautography of the immunoprecipitate after electrophoresis on a gradient slab gel revealed the same pattern of microheterogeneity as usually seen with the purified enzyme.[2] If the original antigen had contained impurities, one might have expected multiple proteins in the immunoprecipitate, but not the same quantitative distribution. Since the cells used for this experiment were washed with 1 M KCl, treated with phenylmethanesulfonyl fluoride, and generally handled to minimize proteolysis, the possibility is raised that the limited proteolysis of amidotransferase occurs in vivo. Since amidotransferase is known to be degraded in stationary cells[2] (Switzer et al., 1979a,b), it is possible that such limited proteolysis is related to the normal degradation process.

B. subtilis amidotransferase shares with the amidotransferase from human (Holmes et al., 1973) and avian sources (Rowe & Wyngaarden, 1968) a tendency to exist in equilibrium between tetrameric and dimeric states. The human enzyme has been reported on the basis of indirect evidence to be an iron–sulfur enzyme (Itakura & Holmes, 1979). The avian enzyme contains iron but has been reported not to contain inorganic sulfide. An interesting contrast is provided by the amidotransferase from *Escherichia coli*, which contains no iron–sulfur center and is probably a tetramer with a subunit M_r of 57 000 (Messenger & Zalkin, 1979). Holmes et al. (1973) have proposed that human amidotransferase is regulated by interconversion between an active P-Rib-PP-stabilized small (M_r 133 000) form and an inactive large (M_r 270 000) form, whose formation is promoted by allosteric nucleotide inhibitors. Although P-Rib-PP and nucleotides also alter the quaternary structure of *B. subtilis* amidotransferase, there is no such simple correlation between activity and quaternary structure. For example, the potent inhibitor AMP promotes formation of the dimer, whereas pairs of synergistically inhibitory nucleotides favor the tetramer (Figure 3). It seems, therefore, that the allosteric regulation of *B. subtilis* amidotransferase must be mediated by conformational changes in the protein that are independent of the state of aggregation.

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[2] M. Ruppen, unpublished experiments.

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