Evidence for a Tetranuclear Iron-Sulfur Center in Glutamine Phosphoribosylpyrophosphate Amidotransferase from Bacillus subtilis*

(Received for publication, December 17, 1979, and in revised form, March 31, 1980)

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Glutamine phosphoribosylpyrophosphate amidotransferase from Bacillus subtilis is an unusual enzyme because it contains an essential iron-sulfur center but does not catalyze an obvious oxidation-reduction reaction. In this communication, results of revised sulfide analyses, iron-sulfur cluster displacement studies, and Mössbauer spectroscopy are presented that lead to the conclusion that the native enzyme contains a tetranuclear [4Fe-4S] center in a diamagnetic state.

The discovery of iron-sulfur centers in glutamine phosphoribosylpyrophosphate amidotransferase (1) and acinitase (2-4), enzymes that do not catalyze obvious oxidation-reduction reactions, requires a re-examination of current ideas concerning the function of iron-sulfur centers in enzymes. A necessary first step is to establish whether the structure of the iron-sulfur centers in these enzymes is like the binuclear and tetranuclear centers found in other proteins (5). In their initial description of the purified amidotransferase from Bacillus subtilis, Wong et al. (1) reported an iron and inorganic sulfide content of 3 and 2 atoms/subunit, respectively, which left the structure of the iron-sulfur center uncertain. In this communication, we present evidence, based on revised sulfide analyses, cluster displacement studies, and Mössbauer spectroscopy, that native amidotransferase contains a tetranuclear iron-sulfur center in a diamagnetic state.

EXPERIMENTAL PROCEDURES

Amidotransferase was purified from B. subtilis and assayed by procedures reported elsewhere (1, 6). For preparation of 57Fe-containing amidotransferase B. subtilis strain 60164 was grown on a defined medium containing the following (per liter): 5 g of glucose, 2 g of (NH4)2SO4, Mann, enzyme grade) 0.28 g of K2HPO4, 0.12 g of KH2PO4, 1 g of sodium citrate-2H2O, 0.2 g of MgSO4·7H2O, 1 g of casamino acids (Difco B230), 15 mg of adenine, 5 mg of thiamin-HCl, 10 mg of CaCl2, 5 mg of MnCl2, and 0.2 mg of 57Fe (prepared by dissolving 57Fe metal, >90% 57Fe from Oak Ridge National Laboratories, in concentrated HCl). The pH of the medium was maintained at 6.5 to 6.8 by addition of KOH during growth. Iron content was determined by complexation with 2,4,6-tripyridyl-s-triazine using a modification (6) of the procedure of Fischer and Price (7) and by measurements with a Perkin-Elmer 303 atomic absorption spectrophotometer employing Fe(NO3)3·9H2O in 0.2 N HNO3 as a standard. Inorganic sulfide was measured using the method of King and Morris (8) with the following modifications. Assays were performed in 1.5-ml self-capping polypropylene centrifuge tubes with a final fluid volume of 1.0 ml. Incubation of protein samples in the alkaline zinc reagent was increased to 2 h, during which time the stopped tubes were blended on a Vortex mixer for 5 to 10 s every 30 min. Sulfide content was corrected for S2- released from cysteinyI residues as determined from identical treatment of amidotransferase apoprotein (1). Na2S was used as a standard as previously described (1). The absorbance yield at 670 nm was 32.3 cm-1 μmol-1 (1.0 ml final volume), which is about 10% higher than most previously reported values (8). Amidotransferase concentrations were determined from an extinction coefficient of 278 nm based on dry weight (1); a molecular weight for the subunit of 50,000 was used.

The methods used for displacement (extrusion) of Fe-S clusters have been described in detail (4, 9, 10). Amidotransferase was examined using both (A) the simple displacement method (9) with thiophenol, and (B) the double displacement reaction (4, 10), in which the Fe-S chromophores are initially removed with o-xyl(SH)2', followed by ligand substitution with thiophenol. In both cases, quantitation was by spectrophotometric methods (4, 9), using a Cary 17 spectrophotometer. Samples of amidotransferase (7.5 to 10 mg/ml) in 0.20 ml of 250 mM Tris-HCl buffer (pH 9.0 (A) or 8.0 (B)) were treated at room temperature with 0.80 ml of 12.5 mM thiophenol (A) or 12 mM o-xyl(SH)2', (B) in HMPA. The reactions were monitored spectrophotometrically in the double-septom-seal cuvettes described previously (9), until the reaction reached completion (~1 h for thiophenol, <10 min for o-xyl(SH)2'). In the case of Method B, 6.25 μl of thiophenol was injected at this point, and the reaction was monitored spectrophotometrically again until completion (<10 min). Control reactions with spinach ferredoxin and Clostridium pasteurianum ferredoxin (both from Sigma Chemical Co.) were carried out in an analogous manner. Extinction coefficients determined in the control experiments agreed well with those reported previously (4, 9).

The Mössbauer samples were immersed in liquid nitrogen for freezing and storage. They were transferred for measurement to a variable temperature cryostat (Janis Research Corp.) or to a 4.2 K cryostat equipped with a superconducting magnet. The Mössbauer spectrometer was of the constant acceleration type. All Doppler shifts, in particular the chemical shifts, δ, are referred to metallic iron at room temperature.

RESULTS

Iron and Sulfide Analysis—The unconventional values of 3Fe and 2S2- atoms/amidotransferase obtained in previous studies (1) prompted us to examine these values in several different preparations of the enzyme by a variety of procedures. Table I presents some typical results. The previous iron analyses by chemical and neutron activation analysis have been confirmed with atomic absorption spectroscopy. A range from 2.6 to 3.7 atoms of iron/subunit has been observed in various preparations. The procedure of King and Morris (8) for S2- analysis was found to be extremely sensitive to the amount of mixing and volume of air above the sample during the incubations at each step. When these were minimized, as described under "Experimental Procedures," the values in

* The abbreviations used are: o-xyl(SH)2', o-xyl(SH)2'-dithiol; HMPA, hexamethyldisilamidophosphoramide; HiPP, high potential iron protein.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 13, Issue of July 10, pp. 6007-6013, 1980
Printed in U.S.A.
Table I were obtained. The moles of S²⁻/amidotransferase subunit was as high as 3.3 and was generally, but not always, within 5% of the iron content. Although no preparation of the enzyme was found to contain 4 atoms of Fe and S²⁻ per subunit, the finding of equimolar Fe and S²⁻ at values greater than 3 suggests that the native enzyme contains a tetranuclear Fe-S center.

Table 1

| Sample   | Spectrophotometric assay (8) | Atomic absorption | Inorganic sulfide content |
|----------|-----------------------------|-------------------|--------------------------|
|          | atoms/subunit               | atoms/subunit     |                          |
| Preparation 35 | 2.65 ± 0.05                | 2.58 ± 0.05       | 2.52 ± 0.05              |
| Preparation 36 | 3.28 ± 0.01                | 3.22 ± 0.05       | 3.20 ± 0.05              |
| Preparation 44 | 3.88 ± 0.06                |                   | 3.12 ± 0.09              |

The optical spectrum of amidotransferase after sequential treatment with o-xyl(SH)₂ and thiophenol in 80% v/v HMPA is compared in Fig. 1 to the spectra of samples of C. pasteurianum and spinach ferredoxins under identical conditions, all at the same total iron concentration. Since C. pasteurianum ferredoxin is known to contain 2 [4Fe-4S] cores and spinach ferredoxin to contain a single [2Fe-2S] core, the results clearly establish that the Fe-S prosthetic group of amidotransferase is extruded as a tetranuclear cluster. Similar spectra were obtained upon treatment of amidotransferase with thiophenol alone in HMPA, but the displacement reaction was much slower, requiring ≥60 min for completion. Final concentrations of thiophenol above 20 mM, while increasing the rate of displacement, also caused some turbidity, apparently due to precipitation of apoprotein. The optical spectrum of amidotransferase treated with o-xyl(SH)₂ in 80% v/v HMPA (not shown) was very similar in both peak position and overall shape to that obtained with C. pasteurianum ferredoxin, and bore no similarity to the characteristic spectrum (4) obtained with spinach ferredoxin, further indicating the presence of a [4Fe-4S] cluster in amidotransferase.

Quantitation of released [4Fe-4S] clusters using known (4, 9) extinction coefficients gave 0.74 and 0.69 [4Fe-4S] clusters under a variety of experimental conditions.

In view of the very recent discovery of three-iron centers (13-15), the evidence for and against such a cluster in amidotransferase must be closely examined. Accepting the facts that (i) the iron sites in native amidotransferase are not distinguishable by Mössbauer spectroscopy, (ii) that they display isomer shifts and quadrupole splittings halfway between the values typical for ferrous and ferric high spin iron in tetrahedral sulfur coordination, and (iii) that they couple to a total spin of zero, ΣS = 0, the only plausible spin assignments are S₁ = S₂ = S₃ = 2 for a tetranuclear, and S₁ = S₂ = 5/2, S₃ = 2 for a trinuclear cluster. The former is the accepted assignment for reduced HiPIP, the latter corresponds to the reduced state of the three-iron centers that have been studied to date (13, 14), which are

![Fig. 1. Optical spectra of (A) amidotransferase (1.93 mg/ml), (B) C. pasteurianum ferredoxin (14.4 µM), and (C) spinach ferredoxin (57.5 µM) in 80% v/v HMPA containing 50 mM Tris-HCl, pH 8.0, 9.6 mM o-xyl(SH)₂, and 60 mM thiophenol, prepared as described (Method B) in the text. The total iron concentration is 115 µM in each case. Optical path length: 1.0 cm.](image-url)
DISCUSSION

The combined weight of evidence presented above clearly indicates that native amidotransferase contains a \([4\text{Fe}-4\text{S}]\) cluster as its prosthetic group. The iron and sulfide analyses of 3 and 2 atoms/subunit, respectively, reported previously (1) did not agree with this conclusion. The present studies have shown, however, that seemingly minor variations in the sulfide analysis procedure can lead to substantial underestimation of the sulfide content. We believe that present analyses of up to 3.3 sulfides/subunit are more nearly correct, because the procedure used gives both the highest color yield with standard sulfide solution and the highest sulfide content with amidotransferase.

Although the consistently high iron and sulfide content suggested the presence of \([4\text{Fe}-4\text{S}]\) clusters in less than stoichiometric amounts, the possibility that amidotransferase might contain two \([2\text{Fe}-2\text{S}]\) clusters was examined using the cluster extrusion (displacement) method. Initial results with thiophenol in HMPA gave clear evidence for the presence of tetranuclear Fe-S clusters in amidotransferase; the quantity of \([4\text{Fe}-4\text{S}]\) centers agreed very well with the measured iron and sulfide contents. Because the reaction with thiophenol was sluggish, we also examined the reaction of amidotransferase with o-xyl(SH)\(_2\), a reagent known to stabilize \([2\text{Fe}-2\text{S}]\) centers markedly (9). Since this also caused rapid release of the Fe-S chromophores, this method should minimize the possibility that \([2\text{Fe}-2\text{S}]\) centers are present in native amidotransferase, but are converted to \([4\text{Fe}-4\text{S}]\) clusters during removal from the protein. Results using both o-xyl(SH)\(_2\) and the thiophenolate derivatives resulting from sequential ligand exchange reaction, which are spectroscopically more characteristic, were in excellent agreement with the presence of a \([4\text{Fe}-4\text{S}]\) center in most of the isolated enzyme molecules. When the Azotobacter vinelandii ferredoxin, which has been recently reported to contain a novel \([3\text{Fe}-3\text{S}]\) center (13, 15), is treated with o-xyl(SH)\(_2\), the center is extruded as a \([2\text{Fe}-2\text{S}]\) cluster. Furthermore, the clusters extruded from the A. vinelandii ferredoxin with thiophenol show distinctive spectral features (cf. Fig. 12 of Ref. 9) which were not detected in the present case. Thus, the results of our cluster displacement studies, like the Mössbauer data, argue against the presence of a \([3\text{Fe}-3\text{S}]\) cluster in amidotransferase. Whether the \([4\text{Fe}-\text{4S}]\) centers are lost from a variable fraction of amidotransferase molecules during isolation or the cells normally produce a small amount of apoprotein molecules is not known. Isolation of amidotransferase from cells grown on limiting amounts of iron, however, also yielded enzyme containing about 3 atoms of iron/subunit.

The Mössbauer data indicate that native amidotransferase is diamagnetic, in good agreement with preliminary EPR studies.\(^2\) The values of the Mössbauer parameters agree very well with those expected for a \([4\text{Fe}-4\text{S}]\) cluster in the +2 \((C^\text{II})\) oxidation state, with an average iron valence of +2.5 and the electronic spins of the iron atoms coupled and delocalized over four essentially equivalent iron atoms (12). Ferredoxins and high potential iron proteins in this oxidation state can be readily reduced or oxidized by 1 electron/4 iron ions to yield centers with characteristic EPR and Mössbauer spectra. Such behavior has been difficult to observe in the case of amidotransferase, but preliminary experiments now indicate that it may be possible to carry out limited, reversible oxidation and reduction of the Fe-S cluster of the enzyme.\(^4\) Characterization of such reactions may allow a more detailed comparison to be made between the \([4\text{Fe}-4\text{S}]\) cluster of amidotransferase and the more thoroughly studied \([4\text{Fe}-4\text{S}]\) clusters of other proteins. The iron-sulfur center in amidotransferase is essential for enzymatic activity, because reaction with O\(_2\) (16) or o-phenanthroline (1) causes complete inactivation. Activity can be partially restored by incubation with sulfide, ferrous ion, and thiols. Whether the center participates directly or indirectly in catalysis remains unknown; it seems unlikely, however, that the \([4\text{Fe}-4\text{S}]\) center is oxidized or reduced during catalysis. The only other enzyme that catalyzes a non-oxidation-reduction reaction and contains iron and acid-labile sulfide, aconitase, has been claimed to contain a \([2\text{Fe}-2\text{S}]\) center (4), but more recent evidence suggests the presence of a \([3\text{Fe}-3\text{S}]\) cluster (13). These results suggest that Fe-S centers may have general importance in other than oxidation-reduction reactions.

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\(^2\)The reason for the rapid reaction with o-xyl(SH)\(_2\) is not clear, but similar results have been reported by others (10). A possibility is that the dithiol, acting as a lipophilic analog of dithiothreitol, reduces intraprotein disulfide bonds, causing rapid unfolding and exposure of the Fe-S prosthetic groups.

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