The PLP-dependent biotin synthase from *Escherichia coli*: mechanistic studies

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Abstract Biotin synthase (BioB), an iron–sulfur enzyme, catalyzes the last step of the biotin biosynthesis pathway. The reaction consists in the introduction of a sulfur atom into two non-activated C–H bonds of dethiobiotin. Substrate radical activation is initiated by the reductive cleavage of *S*-adenosylmethionine (AdoMet) into a 5′-deoxyadenosyl radical. The recently described pyridoxal 5′-phosphate-bound enzyme was used to show that only one molecule of AdoMet, and not two, is required for the formation of one molecule of biotin. Furthermore 5′-deoxyadenosine, a product of the reaction, strongly inhibited biotin formation, an observation that may explain why BioB is not able to make more than one turnover. However this enzyme inactivation is not irreversible.

Key words: Biotin synthase; Iron–sulfur cluster; Sulfur donor; 5′-Deoxyadenosine; Enzyme mechanism

1. Introduction

Biotin synthase (BioB) catalyzes the last step of the biotin biosynthetic pathway [1]. The reaction consists in the introduction of a sulfur atom into dethiobiotin (DTB), thus requiring activation of two C–H bonds (Scheme 1). The enzyme from *Escherichia coli*, a homodimer of 76 kDa, has been the most extensively studied BioB so far [2,3]. However, whereas there is a general agreement for the presence, on each polypeptide chain of this enzyme, of an oxygen-sensitive (4Fe–4S) cluster [4–6], ligated by three cysteines of a conserved CXXXCXXC box and a fourth still not identified ligand [7–9], there are still inconsistencies as far as the requirement for other cofactors is concerned. Indeed, comparable enzyme activities in vitro have been recently obtained using purified preparations containing in each polypeptide chain either two iron–sulfur centers, the (4Fe–4S) one and an additional (2Fe–2S) cluster (model 1) [10–12], or only the (4Fe–4S) cluster and one protein-bound pyridoxal 5′-phosphate (PLP) (model 2) [13]. In the first case, the sulfur atom incorporated into biotin is proposed to derive from the sulfide ions of the (2Fe–2S) center [10–12,14] whereas, in the second case, it is proposed to derive from a protein-bound persulfide, generated during PLP-dependent desulfuration of free cysteine [13]. Which combination is operating in vivo remains to be found.

On the other hand there is a general agreement on the function of the (4Fe–4S) cluster in both models. It is proposed to serve as a catalyst for the reduction and the cleavage of *S*-adenosylmethionine (AdoMet) by enzymatically reduced flavodoxin (Fldx) into a putative 5′-deoxyadenosyl radical (Ado°) required for the abstraction of hydrogen atoms of DTB [15]. Since the formation of one molecule of biotin implies the conversion of two C–H bonds, at C-9 and C-6 positions of the substrate, into C–S bonds, it is generally inferred that two Ado° radicals are required and thus two molecules of AdoMet are consumed for one single turnover [16,17]. The resulting carbon radicals then acquire a sulfur atom either from the (2Fe–2S) center (model 1) or from a persulfide (model 2). In this paper, we report experiments allowing determination of the AdoMet/biotin ratio in the case of the PLP-dependent system and discuss a novel mechanistic scenario.

Another important issue that we address in this paper deals with the low BioB activity in vitro. So far, the largest reported activities, with the purified enzyme from *E. coli* and well-defined in vitro assay mixtures, rarely exceed 1 nmol of biotin per nmol of monomer. This failure to obtain multiple turnovers has sometimes been put forward to propose that BioB is not an enzyme but instead a reactant [14]. On the other hand, BioB from *Arabidopsis thaliana* was recently shown to support multiple turnovers [18]. We thus reasoned that an irreversible inactivation of the *E. coli* enzyme, by some unknown mechanisms, might occur in vitro or that essential cofactors, not identified yet, be missing. Here we demonstrate that in fact 5′-deoxyadenosine (AdoH), a product of the reaction, is a very strong inhibitor, thus providing a simple explanation for the lack of multiple turnovers and strongly suggesting that BioB is a true enzyme.

2. Materials and methods

2.1. Materials

All chemicals were of reagent grade and obtained from Sigma-Aldrich Chemical Co. or Fluka unless otherwise stated. AdoMet and methionine were from Boehringer Mannheim. PLP was from Interchim. 5-Deazaflavin, Fldx and Fldx reductase were available in our laboratory.

2.2. Preparation of BioB

BioB was overexpressed in BL21(DE3) *E. coli* strain carrying pKH200 plasmid as already described [5,13]. Overexpression and purification procedures were standard [5,13]. ApoBioB protein was prepared and reconstituted with $Fe(NH_4)_2(SO_4)_2$ and Na_2S as already

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Abbreviations: BioB, biotin synthase; PLP, pyridoxal 5′-phosphate; AdoMet, *S*-adenosylmethionine; AdoH, 5′-deoxyadenosine; DTT, dithiothreitol

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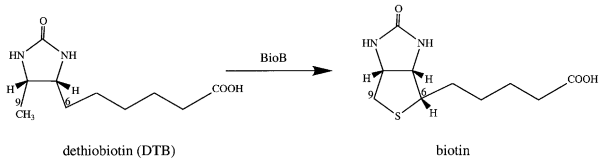


Scheme 1. The reaction catalyzed by BioB.

described [5,13]. The reconstituted protein was treated with 2 mM EDTA for 30 min and desalted over Sephadex G-25 to remove excess iron and sulfide.

2.3. BioB activity

The activity was assayed from the amount of biotin formed from DTB. The standard reaction mixture in a final volume of 50 μl 0.1 M Tris–HCl, pH: 8, 30 mM KCl contained 1.7 nmol BioB, 1 equivalent of PLP, 400 μM DTB, 1 mM dithiothreitol (DTT), 150 μM AdoMet, 20 μM Fldx, 4 μM Fldx reductase, 1 mM NADPH, 2 mM cysteine. The reaction was monitored at 37°C. At time intervals (from 5 to 180 min) an aliquot was withdrawn and the reaction stopped by the addition of 10% (v/v) 1 M TCA. After centrifugation, the supernatant was divided into three portions, for biotin, alanine and methionine assay, respectively.

2.3.1. Biotin determination. Biotin was measured by a microbiological method using *Lactobacillus plantarum* [19], using a calibration curve in each experiment. All the data presented in this paper represent the average of at least duplicate experiments.

2.3.2. Alanine and methionine determination. The portions, containing 5 nmol of BioB, of the supernatant prepared as described above were dried on a speed-vac and the residue dissolved into 150 μl of a citrate buffer, pH: 2.2. For each amino acid a ninhydrin-derivative was generated and analyzed at 570 nm by HPLC on a 7300 Beckman apparatus working with an ion exchange column S101036 calibrated with pure amino acid standards according to a published procedure [20].

2.4. Analysis

Protein concentration (by monomer) was determined by the method of Bradford [21] standardized with bovine serum albumin. A correction factor of 1.1 was applied according to dry weight measurements from amino acid analysis. Protein-bound iron [22] and labile sulfide [23] were determined according to standard procedures.

2.5. Reaction of the reduced 4Fe–4S cluster with AdoMet

All the experiments were done under anaerobic conditions inside the glove box (<2 ppm O_2, 18°C). BioB (160 μM) in 400 ml of 0.1 M Tris–HCl, pH: 8.0, 30 mM KCl, was first irradiated in the presence of 5-deazaflavin (50 μM) and AdoH (500 μM). Reduction was monitored by spectrophotometry directly inside the box. After 40 min reduction, one portion of the reduced protein solution was shielded from light using an aluminum foil and complemented with AdoMet (500 μM), whereas the second portion did not receive AdoMet. After 30 min incubation, both mixtures were assayed for methionine and also for the presence of the reduced cluster by EPR spectroscopy. Spectra were recorded on a Bruker EMX (9.5 GHz) EPR spectrometer equipped with an ESR 900 Helium flow cryostat (Oxford Instruments). Double integrals of EPR signals and spin concentration were obtained through the Win-EPR software using the spectrum of a 1 mM Cu(EDTA) standard recorded under non-saturated conditions.

3. Results

3.1. Multiple turnovers in BioB

Production of biotin from DTB catalyzed by BioB was assayed at 37°C under anaerobic conditions in the presence of AdoMet, DTT, cysteine, PLP and the NADPH/Fldx reductase/Fldx system. Optimization of the assay using 30–40 μM BioB and 1 equivalent of PLP led to the following concentrations: 0.15 mM AdoMet, 0.5–1 mM DTT, 0.4 mM DTB, 2 mM cysteine, 1 mM NADPH, 20 μM Fldx and 4 μM Fldx reductase (data not shown). In all the experiments reported here BioB is the preparation obtained by reconstitution of the apoprotein with an excess of iron and sulfide and containing 3.5–4 Fe per monomer in the form of a 4Fe–4S cluster after desalting on Sephadex G-25 [5]. Under these conditions, the maximal amount of biotin is 0.7–1 mol per mol of monomer. In the experiment shown in Fig. 1, it is observed that biotin formation proceeded linearly with time for about 40 min, with an initial reaction rate of about 1 mol biotin/mol BioB monomer/h, and levelled off then, to reach 0.7 mol biotin per BioB monomer.

Whether this inability of the enzyme to allow multiple turnovers was reversible or not was tested in experiments in which, after a first run, BioB was isolated from the assay mixture by filtration on Nanosep 30 (Millipore) and assayed again for activity with a freshly prepared assay mixture. The results in Fig. 1 clearly show that such a desalted BioB preparation recovered almost full activity. During the second run, biotin formation indeed occurred with initial rates and total yields comparable to those obtained after the first run. In conclusion, the lack of multiple turnovers always observed with BioB from *E. coli* is not due to an irreversible inactivation of the enzyme. The experiment suggests instead that a compound which accumulates during the reaction behaves as an efficient but reversible inhibitor. It thus tells that BioB has the potential to work as a true enzyme.

3.2. Inhibition of BioB by AdoH

Under standard assay conditions, the following compounds have been tested as possible inhibitors: $NADP^+$, which derives from the oxidation of NADPH, methionine and AdoH, which are produced by cleavage of AdoMet. Whereas addition of the two first compounds had no effect on the activity (data not shown), AdoH proved to be a very efficient inhibitor since, as shown in Fig. 2, almost 90% of the activity was lost upon addition of 1 equivalent of AdoH with regard to BioB. Full inhibition was observed with 1.5 equivalents.

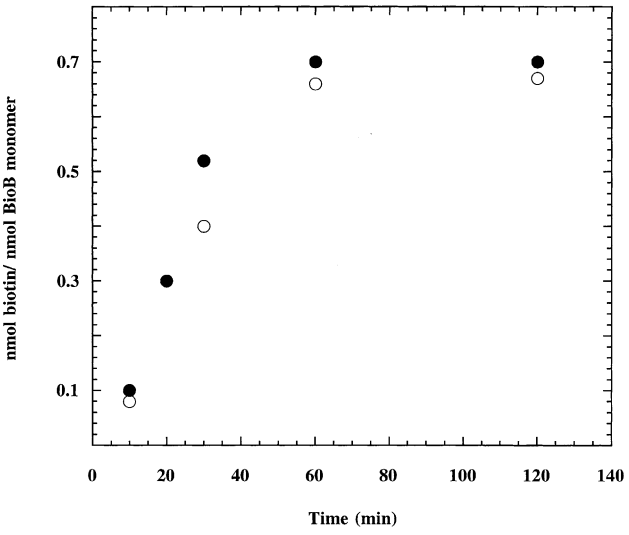


Fig. 1. Time course for the formation of biotin. BioB (35 μM) was incubated with 400 μM DTB, 1 mM DTT, 150 μM AdoMet, 20 μM Fldx, 4 μM Fldx reductase, 1 mM NADPH, 2 mM cysteine, and 1 equivalent of PLP at 37°C. At the indicated times, an aliquot was removed and assayed for biotin (●). After 120 min the reaction mixture was filtered and BioB recovered for a second incubation with a freshly made enzyme assay mixture. The reaction was again monitored from biotin formation (○).

The iron–sulfur cluster of BioB can be prepared in the $S = 1/2$ EPR-active reduced form and its anaerobic reaction with AdoMet can be monitored from both the formation of methionine and the decay of the intensity of the EPR signal characteristic for the cluster [5,15]. Addition of AdoH, 1–3 equivalents with regard to BioB, did not result in an inhibition of that reaction (data not shown). In a control experiment the reduced cluster was shown to be stable in the presence of similar excesses of AdoH under anaerobic conditions.

3.3. Stoichiometric formation of biotin, methionine and alanine

In the PLP-dependent in vitro system, biotin synthesis requires both AdoMet reductive cleavage into methionine and a 5′-deoxyadenosyl radical for activation of the substrate and cysteine desulfuration into alanine and a persulfide as a source of sulfur. The reaction can thus be monitored not only from the formation of biotin, as usually done, but also in parallel from that of methionine and alanine [13,15]. In the experiment shown in Fig. 3 the three parameters were determined concommitantly during the course of biotin formation under standard conditions. These results show two features. First, in the first step of the reaction, when biotin was produced, both methionine and alanine were formed in equimolar amounts with regard to biotin. Second, in the second step of the reaction, when no further production of biotin could be observed, methionine formation also stopped, thus perfectly following biotin, whereas cysteine desulfuration, as monitored by alanine formation, continued to proceed, at a slightly increased rate.

4. Discussion

BioB from *E. coli* has been the subject of intense studies in the last years. As a matter of fact this enzyme displays a number of intriguing properties which are still resistant to interpretation. The results presented here provide novel insights into some of these important questions in relation to BioB activity and mechanism. The enzyme form used in this study is the one containing a (4Fe–4S) cluster and PLP that was discovered in our laboratory [13].

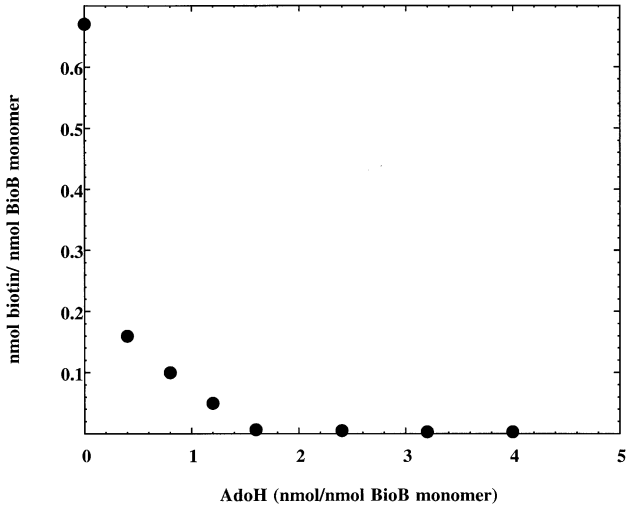


Fig. 2. Biotin formation activity as a function of AdoH concentration. Under conditions described in Fig. 1 using BioB (35 μM monomer) biotin formation was assayed in the presence of increasing concentrations of AdoH and after 90 min reaction at 37°C.

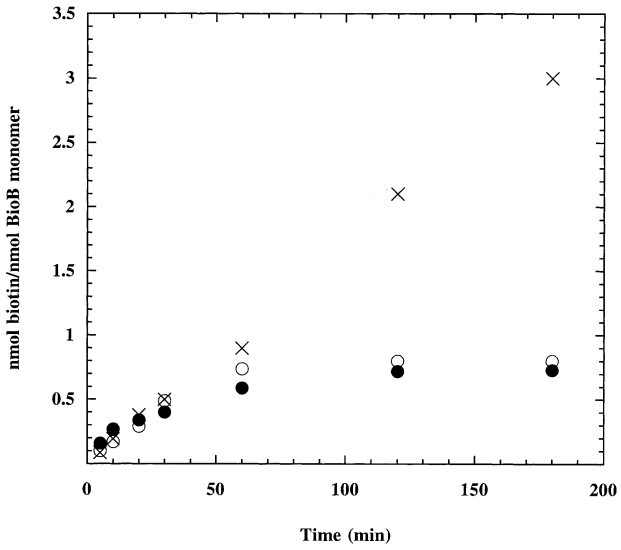
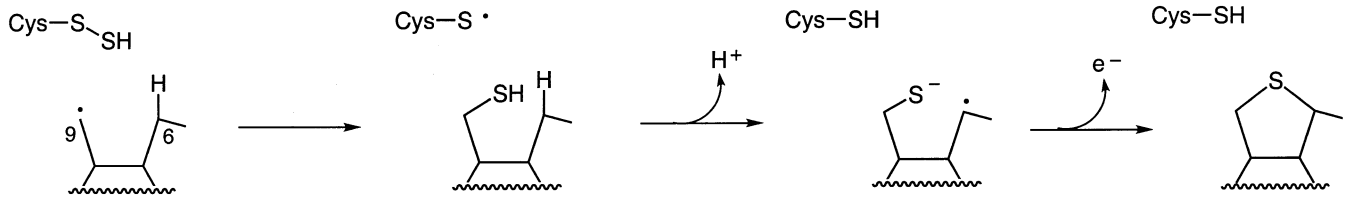


Fig. 3. Time course for the formation of biotin, alanine and methionine. BioB (35 μM) was incubated under conditions described in Fig. 1. At the indicated times, biotin (○), alanine (×) and methionine (●) were assayed as described under Section 2.

The first question deals with the inability of the enzyme to support multiple turnovers. It has been repeatedly suggested either that BioB was irreversibly inactivated during the reaction by some undefined mechanism or that an important cofactor/protein was missing in the in vitro assay mixture. In the second case the requirement for a machinery involved in the reconstitution of an iron–sulfur center was frequently discussed in the context of the recent hypothesis that an iron–sulfur center was the sulfur donor implying the loss of a sulfur bridge at each turnover. The most extreme suggestion was that BioB was not an enzyme but a reactant [14].

In fact our results suggest an alternative hypothesis. BioB is not irreversibly inactivated and is able to make several turnovers, but only if it is isolated from the reaction mixture after each cycle and incubated with a freshly prepared assay mixture for the next cycle. A simple interpretation is that a product accumulating during the reaction displays strong inhibitory effects, which can be reversed by its removal. An obvious candidate is AdoH since it is, among the various reaction products, the only one that inhibits the enzyme (Fig. 2). AdoH is produced by the reductive cleavage of AdoMet via an intermediate 5′-deoxyadenosyl radical which then abstracts a hydrogen atom on the substrate and thus activates it for sulfur insertion. The reason why AdoH is so efficient as an inhibitor of BioB is not clear at that stage. Two key chemical processes of the reaction, namely the electron transfer from the reduced cluster to AdoMet and the desulfuration of cysteine, are indeed not affected by the presence of AdoH. On the other hand the fact that methionine formation levels off in parallel with biotin suggests that the first steps of the reaction are critical. AdoH could for example interfere with the electron transfer from NADPH to the iron–sulfur cluster. Thus further experiments are required to better define the inhibition mechanism and to understand which particular conditions, not fulfilled yet, are required for limiting this inhibitory effect of AdoH in an in vitro assay and to make BioB catalytically active. We indeed speculate that this is an artefact of the in vitro conditions used so far and that in vivo BioB manages to



Scheme 2. Hypothetical mechanism for sulfur insertion in DTB.

avoid this inhibition as suggested from experiments carried out with BioB from *A. thaliana* [18].

The second question concerns the number of AdoMet molecules to be cleaved for the production of one biotin molecule. This ratio can be determined by measuring in the same experiment the amount of biotin and that of either methionine or AdoH since the two compounds are formed in equimolar amounts. In previous experiments with well-defined assays Marquet et al. reported 2.8–2.9 AdoH and 2.6–3.1 methionine/biotin [17]. Since the theoretical maximal amount of AdoMet per biotin is 2, one for each C–H bond to be cleaved, these authors had to conclude that their results were indicating an AdoMet/biotin ratio of 2 and to speculate that under their assay conditions an unspecific formation of AdoH was occurring. With this drawback and the fact that the enzyme activities in these experiments were determined with preparations lacking PLP and furthermore quite weak (0.03–0.1 nmol biotin per nmol monomer), determination of the stoichiometry of the reaction was worth to be repeated with our PLP–BioB preparations. Our data (Fig. 3) clearly show that in the first phase of the reaction during which biotin is produced one, and not two, molecule of methionine is formed per molecule of biotin formed. This thus challenges proposed mechanisms in the literature which generally imply that two 5′-deoxyadenosyl radicals are generated and used, one for abstraction of each C–H bond at C-9 and at C-6 carbons of DTB. At this stage it is difficult to explain the inconsistency with Marquet's results [17]. However, one cannot exclude that differences in BioB preparations account for it. The additional observation that one alanine from cysteine was formed per biotin nicely fits with the requirement for one sulfur atom per biotin and this correlation further supports the importance of the cysteine desulfurase activity of BioB for biotin synthesis [13].

In Scheme 2 is shown a speculative mechanism accounting for the biotin:methionine:alanine 1:1:1 ratio. Four oxidizing equivalents are required for the conversion of DTB to biotin. It is proposed here that they are provided by one 5′-deoxyadenosyl radical (1 eq.), one persulfide (2 eq.) and a fourth still unidentified equivalent. The 5′-deoxyadenosyl radical, derived from the one-electron reduction of AdoMet, abstracts a H atom at position 9 of DTB and is converted into AdoH. In parallel sulfur from free cysteine is transferred to the protein generating a reactive protein-bound persulfide species. As shown in Scheme 2, the substrate C-9 radical couples to the terminal sulfur of the persulfide giving rise to a thiyl radical and 9-mercaptodethiobiotin. The thiyl radical then abstracts a H atom at C-6 and ring closure, leading to formation of biotin, together with the release of one electron. Abstraction of the C-6 hydrogen atom by a cysteinyl radical is thermodynamically unfavorable, considering the bond dissociation energies of S–H (93 kcal mol^{-1}) and C–H bonds (100 kcal mol^{-1}). However this step is likely to be driven by coupling to the following reaction which is highly energetically favorable. There are several possible candidates as for the electron acceptor in the last step of Scheme 2, for example, among others a ferric center or a disulfide bridge. Once about 1 equivalent of biotin and AdoH is formed, no more biotin can be generated due to the strong inhibitory effects of AdoH and since 1 equivalent of AdoH is enough to almost completely inactivate the enzyme.

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References

[1] Marquet, A., Tse Sum Bui, B. and Florentin, D. (2001) Vitam. Horm. 61, 51–85.
[2] Sanyal, I., Cohen, G. and Flint, D.H. (1994) Biochemistry 33, 3625–3631.
[3] Birch, O.M., Furhmann, M. and Shaw, N.M. (1995) J. Biol. Chem. 270, 19158–19165.
[4] Duin, E.C., Lafferty, M.E., Crouse, B.R., Allen, R.M., Sanyal, I., Flint, D.H. and Johnson, M.K. (1997) Biochemistry 36, 11811–11820.
[5] Ollagnier-de Choudens, S., Sanakis, Y., Hewitson, K.S., Roach, P., Baldwin, J.E., Münck, E. and Fontecave, M. (2000) Biochemistry 39, 4165–4173.
[6] Ugulava, N.B., Gibney, B.R. and Jarrett, J.T. (2000) Biochemistry 39, 5206–5214.
[7] Hewitson, K.S., Baldwin, J.E., Shaw, N.M. and Roach, P.L. (2000) FEBS Lett. 466, 372–376.
[8] McIver, L., Baxter, R.L. and Campopiano, D.J. (2000) J. Biol. Chem. 275, 13888–13894.
[9] Hewitson, K., Ollagnier-de Choudens, S., Sanakis, Y., Shaw, N.M., Baldwin, J.E., Münck, E., Roach, P.L. and Fontecave, M. (2002) J. Biol. Inorg. Chem. 7, 83–93.
[10] Ugulava, N.B., Sacanell, C.J. and Jarrett, J.T. (2001) Biochemistry 40, 8352–8358.
[11] Ugulava, N.B., Sureus, K.K. and Jarrett, J.T. (2002) Biochemistry 40, 8343–8351.
[12] Ugulava, N.B., Gibney, B.R. and Jarrett, J.T. (2002) J. Am. Chem. Soc. 124, 9050–9051.
[13] Ollagnier-de Choudens, S., Mulliez, E., Hewitson, K.S. and Fontecave, M. (2002) Biochemistry 41, 9145–9152.
[14] Tse Sum Bui, B., Florentin, D., Fournier, F., Ploux, O., Mejan, A. and Marquet, A. (1998) FEBS Lett. 440, 226–230.
[15] Ollagnier-de Choudens, S., Sanakis, Y., Hewitson, K., Roach, P., Münck, E. and Fontecave, M. (2002) J. Biol. Chem. 277, 13449–13454.
[16] Escalettes, F., Florentin, D., Tse Sum Bui, B., Lesage, D. and Marquet, A. (1999) J. Am. Chem. Soc. 121, 3571–3578.
[17] Guianvarc'h, D., Florentin, D., Tse Sum Bui, B., Nunzi, F. and Marquet, A. (1997) Biochem. Biophys. Res. Commun. 236, 402–406.
[18] Picciocchi, A., Douce, R. and Alban, C. (2001) Plant Physiol. 127, 1224–1233.
[19] Takana, M., Izumi, Y. and Yamada, H. (1987) J. Microbiol. Methods 6, 237–246.
[20] Moore, A.T. and Stein, W.H. (1963) Methods Enzymol. 6, 819.
[21] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[22] Fish, W.W. (1988) Methods Enzymol. 158, 357–364.
[23] Beinert, H. (1983) Anal. Biochem. 131, 373–378.