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Journal of Molecular Catalysis A: Chemical 95 (1995) 173–178

JOURNAL OF MOLECULAR CATALYSIS A: CHEMICAL

Thermostability and electron transfer activity of the ferredoxin from a thermophilic hydrogen oxidizing bacterium, *Bacillus schlegelii*

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Received 8 June 1994; accepted 20 September 1994

Abstract

The *Azotobacter*-type 7Fe ferredoxin from *Bacillus schlegelii* was active as an electron carrier in the reduction system of cyt. *c* consisting of NADPH, FNR and cytochrome *c* (cyt. *c*). The only [3Fe–4S] cluster in the Fd molecule acts as the active site for the reduction of cyt. *c*. The activity of the ferredoxin once increased by 10, 20 and 20% by heat treatment at 60, 80 and 90°C, respectively. The increasing of the activity is caused by the interconversion of the [4Fe–4S] cluster to the [3Fe–4S] cluster with increasing of the [3Fe–4S] cluster in the concentration.

Keywords: Ferredoxin; Iron–sulphur cluster; *Bacillus schlegelii*

Ferredoxins are typical non-heme iron proteins containing iron–sulfur cluster(s) as the prosthetic groups and classified into five groups based on the type and the number of iron–sulfur cluster(s) in their molecules, i.e., [2Fe–2S], [3Fe–4S], [4Fe–4S], [3Fe–4S][4Fe–4S], and 2[4Fe–4S] ferredoxins [1,2].

The iron–sulfur clusters in ferredoxins are redox active and act as an active site for electron transfer reactions [2]. The redox potential of the iron–sulfur clusters is dependent on the structure of the cluster and the microenvironment around the cluster. For example, *Desulfovibrio gigas* FdI and FdII containing [4Fe–4S] (FdI) and [3Fe–4S] (FdII) cluster in the same polypeptide show the redox potential of −450 mV and −130 mV, respectively [3]. The redox potential of the [3Fe–4S] and the [4Fe–4S] cluster in *Azotobacter*-type 7Fe ferredoxins are lower than that of *D. gigas* Fd. *Azotobacter chroococcum* FdI, for example, has reduction potential of −460 and −645 mV for the [3Fe–4S] and the [4Fe–4S] clusters, respectively [4]. The redox potential of [4Fe–4S] cluster is generally lower than that of [3Fe–4S] cluster by 200–300 mV [1,2].

The interconversion between *D. gigas* FdI and FdII proceeds with changing the structure of the iron–sulfur clusters [5]. The interconversion between *D. gigas* FdI and FdII also causes the change of the oligomeric structure, i.e., a trimer and a tetramer of the same subunit for FdI and FdII, respectively. *D. gigas* FdI and FdII act as

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SSDI 1381-1169(94)00009-3

electron carriers in the phosphoroclastic reaction and the reduction of sulfate, respectively, showing that the interconversion of the iron–sulfur clusters controls the electron transfer reaction [5,6].

Although the interconversion of iron–sulfur cluster in *D. gigas* ferredoxins has been reported to have a physiological meaning, there are some cases of the interconversion without a physiological significance [1]. Oxidative stress such as the treatment of ferredoxins with oxygen or ferricyanide causes the conversion of [4Fe–4S] cluster to [3Fe–4S] cluster [1]. The formed [3Fe–4S] cluster can be converted [4Fe–4S] cluster by the incubation of the protein with Fe^{2+} ion under reduced conditions [7–9].

We reported that a ferredoxin purified from a thermophilic hydrogen oxidizing bacterium, *Bacillus schlegelii*, contained one [3Fe–4S] and one [4Fe–4S] cluster in a protein molecule [10]. The N-terminal amino acid sequence of *B. schlegelii* Fd (Bs-Fd) showed the high homology to *Azotobacter*-type 7Fe ferredoxins. EPR spectroscopy showed that the oxidation state of the [4Fe–4S] and the [3Fe–4S] clusters in Bs-Fd were $[4Fe–4S]^{2+}$ and $[3Fe–4S]^{1+}$, respectively, in the oxidized state [10]. These clusters were reduced by dithionite to be $[4Fe–4S]^{1+}$ and $[3Fe–4S]^{0}$ showing that these clusters in Bs-Fd are redox active.

The optimum growth temperature of *B. schlegelii* is 70°C [11], and the proteins from this bacterium are expected to be thermostable. In this work, we will report the thermostability of Bs-Fd on the electron transfer activity and show that the conversion of [4Fe–4S] cluster to [3Fe–4S] cluster takes place by heat treatment with increasing the electron transfer activity.

1. Material and methods

The cultivation of *B. schlegelii* and the purification of the ferredoxin were done as reported previously [10]. Ferredoxin–NADP oxidoreductase (FNR) and cytochrome *c* from horse heart were purchased from Sigma. NADPH was obtained from Oriental Yeast.

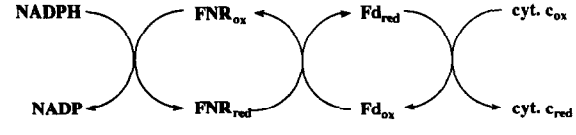
The protein concentration was determined by the Lowry method using bovine serum albumin as a standard [12]. The activity of the ferredoxin as an electron carrier was measured by the initial reduction rate of cytochrome *c* (cyt. *c*) at 30°C in a reaction system containing NADPH, FNR, Fd, and cyt. *c*. The reaction was carried out under argon atmosphere in an optical cell sealed with a rubber septum, and started by adding NADPH solution to the reaction mixture containing FNR, Fd and cyt. *c*. The reactants were degassed in 50 mM of Tris–HCl buffer (pH 8.0) containing 3 mM of EDTA in the final volume of 2 ml. The amount of the reduced cyt. *c* formed was determined by the change of the absorbance at 550 nm.

The Fd dissolved in 50 mM Tris–HCl buffer (pH 8.0) with a concentration of 260 μg/ml was incubated at appropriate temperature for the heat treatment. When the Fd solution was incubated under anaerobic conditions, the solution in a small vial sealed with a rubber septum was degassed with a vacuum pump and then flushed with argon for several cycles before the incubation. A portion of the Fd solution was withdrawn to measure the electron transfer activity at appropriate intervals during the incubation.

EPR spectra were measured on a JEOL FE3XG equipped with a helium flow cryostat (Air Products LTR 3-110). Absorption spectra were measured on a Shimadzu UV-265 equipped with a temperature controller TCC-260.

2. Results and discussion

The activity of Bs-Fd as an electron carrier was measured by the reduction of cyt. *c* in the reaction system shown in Scheme 1. The typical results are



Scheme 1.

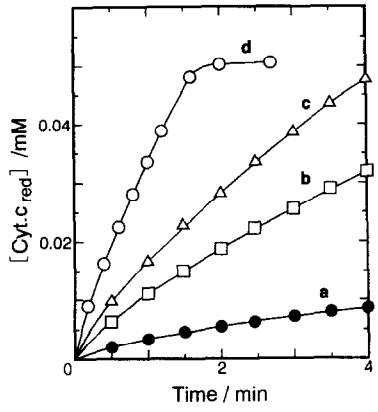


Fig. 1. Time course of the reduction of cyt. *c*. The reaction conditions are [NADPH] = 280 μM, [FNR] = 0.15 μM, [cyt. *c*] = 60 μM, [Fd] = 0 (a), 0.2 (b), 0.4 (c), and 1.0 μM (d). The reactants are dissolved in 50 mM Tris-HCl buffer (pH 8.0).

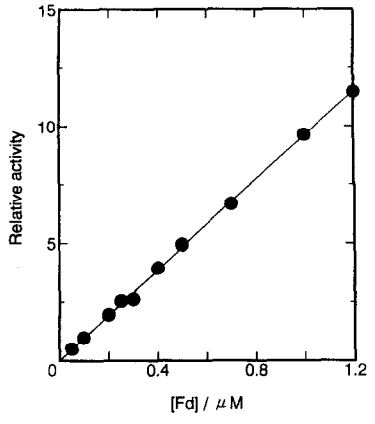


Fig. 2. The dependence of the activity on the concentration of Bs-Fd. The activity is represented as the relative activity. The unity of the relative activity is the reduction rate of cyt. *c* of 3.4 μM min^{-1}.

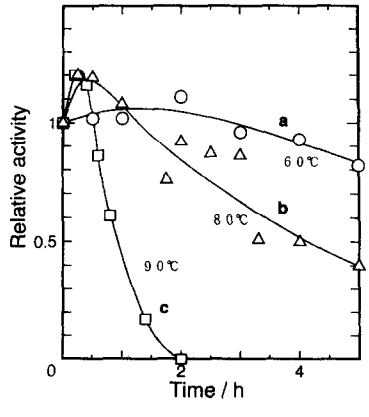


Fig. 3. The effect of aerobic heat treatment on the activity of Bs-Fd. The incubation of Bs-Fd was carried out under aerobic condition at (a) 60, (b) 80, and (c) 90°C.

shown in Fig. 1. In the absence of Bs-Fd, the initial reduction rate of cyt. *c* was 3.4 μM min^{-1}.

The reduction rate of cyt. *c* increased by adding Bs-Fd into the reaction system as shown in Fig. 1 (b–d). The addition of 1 μM of Bs-Fd increased 20-fold the rate of the reduction of cyt. *c* over a reaction mixture only containing NADP, FNR, and cyt. *c*. The initial reduction rate of cyt. *c*, which was obtained by subtracting the initial rate without Bs-Fd from the observed initial rate with Bs-Fd, was proportional to the concentration of Bs-Fd added as shown in Fig. 2. This shows that Bs-Fd is active as an electron carrier in the electron transfer system shown in Scheme 1 and Bs-Fd mediated electron transfer is rate limiting step under the experimental conditions. In the following experiments, 0.2 μM of Bs-Fd was used to measure the activity unless otherwise noted.

Thermostability of Bs-Fd was determined by measuring the residual activity as the electron carrier in the reduction of cyt. *c* after heat treatment at appropriate temperature. The heat treatment was carried out at 60, 80, and 90°C under aerobic conditions. The results are shown in Fig. 3. The strange phenomena were observed in this experiment, i.e., the activity of Bs-Fd once increased by 10, 20, and 20% upon heat treatment at 60, 80, and 90°C, respectively. Heat treatment usually denature proteins spontaneously, but not in this case. The reason why heat treatment caused increasing of the activity will be described later in detail.

Although prolonged heat treatment caused decreasing of the activity beyond the maximum value as shown in Fig. 3, Bs-Fd showed high thermostability. Eighty percent of the original activity was retained after 5 h of heat treatment at 60°C. Increasing the incubation temperature of heat treatment accelerated the decay of the activity. Fifty percent of the original activity, however, was maintained after 4 h and 1 h of heat treatment at 80 and 90°C, respectively.

When Bs-Fd was incubated at 60 and 80°C under anaerobic conditions, the thermostability of Bs-Fd was improved as shown in Fig. 4. The loss of the activity was not observed by heat treatment

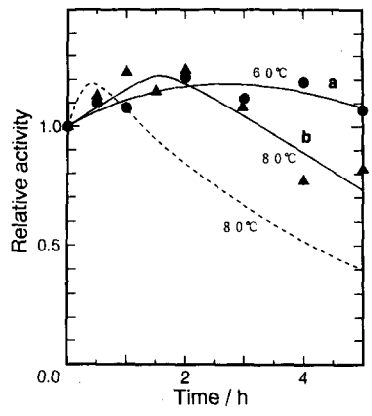


Fig. 4. The effect of anaerobic heat treatment on the activity of Bs-Fd. The incubation of Bs-Fd was carried out under argon atmosphere at (a) 60 and (b) 80°C. The dotted line represents the result of Fig. 3 (b).

at 60°C for 5 h under argon atmosphere. About 75% of the original activity was retained after 5 h incubation at 80°C under the same conditions. When Bs-Fd was incubated under aerobic conditions at 80°C, about 40% of the original activity was retained after 5 h incubation as shown in Fig. 3. The increasing of the activity of Bs-Fd were also observed when heat treatment of Bs-Fd was carried out under argon atmosphere.

The thermostability of *Thermus thermophilus* ferredoxin, which is a thermophilic *Azotobacter*-type 7Fe ferredoxin, was examined by decrease in the absorbance at 400 nm. It was reported that the absorbance decreased to 78 and 71% of the original level after 45 min incubation at 75 and 87°C, respectively [13]. These results show that the thermostability of Bs-Fd is comparable with that of *T. thermophilus* Fd. Sato et al. [13] have suggested the contribution of histidine at position 2 to the heat stability of *T. thermophilus* Fd. This suggestion was derived from the results of Perutz and Raidt [14] for the thermophilic and the mesophilic clostridial ferredoxins. They pointed out that histidine at position 2 formed a hydrogen bond and a salt bridge to contribute to its thermostability [14]. Because *T. thermophilus* Fd has histidine at position 2 [13] and other homologous mesophilic ferredoxins (mesophilic *Azotobacter*-type 7Fe ferredoxins) have not [15–17], Sato et al. [13] proposed the contribution of histidine at position 2 to thermostability of *T. thermophilus* Fd.

The amino acid sequence of Bs-Fd is highly homologous to that of *T. thermophilus* Fd, i.e., 49 amino acids are identical between the two ferredoxins [18]. All of the cysteine residues at positions 8, 16 and 49, and 20, 39, 42 and 45 are conserved, which are deduced to be ligands to the [3Fe–4S] and the [4Fe–4S] cluster, respectively [18]. The second residue of Bs-Fd, however, is tyrosine, not histidine [10,18]. And another homologous thermophilic *Azotobacter*-type 7Fe ferredoxin from *Bacillus acidocaldarius* has phenylalanine at position 2 [19]. These show that histidine at position 2 is not important to thermostability of the thermophilic *Azotobacter*-type 7Fe ferredoxins. The mechanism of the thermostability is not yet obvious, but the study on the mutant Bs-Fd constructed by the site directed mutagenesis is now in progress to clarify the important residues for the thermostability of Bs-Fd.

The activity of Bs-Fd as the electron carrier for the reduction of cyt. *c* increased by heat treatment as described previously. As the iron–sulfur clusters are thought to be active sites for the electron transfer, some modification of the iron–sulfur clusters may occur by heat treatment. To clarify this possibility EPR spectra were measured for the samples incubated at 80°C. The EPR spectra of the oxidized Bs-Fd were shown in Fig. 5. The typical "g = 2.01" signal of $[3Fe–4S]^{1+}$ is observed in the oxidized Bs-Fd [10]. The lineshape of the signal did not change by heat treatment, but the intensity of the signal did. The relative intensity of the signals are summarized in Table 1. The signal of $[3Fe–4S]^{1+}$ increased by 16% in the intensity by the incubation at 80°C for 30 min. Prolonged incubation caused a decrease in the intensity of the signal of $[3Fe–4S]^{1+}$; 96, 73 and 55% of the original intensity were observed in the samples incubated at 80°C for 1, 2 and 4 h, respectively. As described above, the signal of $[3Fe–4S]^{1+}$ in Bs-Fd first increased in the intensity and then gradually decreased by heat treatment.

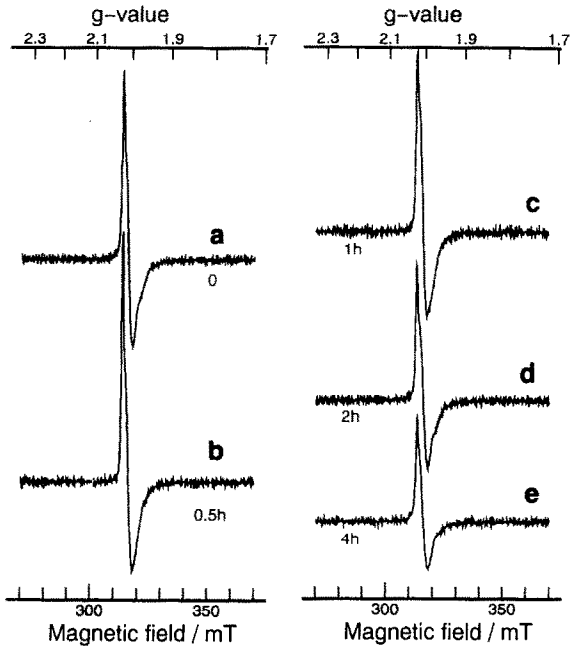


Fig. 5. The EPR spectra of the oxidized Bs-Fd (a) before, and after (b) 0.5, (c) 1, (d) 2, and (e) 4 h of heat treatment under aerobic conditions at 80°C.

Table 1
Relative activity and relative intensity of EPR signal after heat treatment at 80°C

Incubation time/h	Activity	EPR Intensity	
		[3Fe–4S]	[4Fe–4S]
0.5	1.2	1.16	0.82
1.0	1.1	0.96	0.67
2.0	0.8	0.73	0.57
4.0	0.5	0.55	0.26

The EPR spectra of the reduced Bs-Fd were shown in Fig. 6 and the relative intensities are summarized in Table 1. The Bs-Fd was reduced with dithionite after heat treatment under aerobic conditions. The signal of $[4Fe–4S]^{1+}$ is observed in the reduced Bs-Fd [10]. As in the case of the oxidized Bs-Fd, the lineshape of the signal did not change by heat treatment but the intensity of the signal did. The signal of $[4Fe–4S]^{1+}$, however, decreased monotonously in intensity by heat treatment. The relative intensity were 0.82, 0.67, 0.57 and 0.26 for the Bs-Fd incubated at 80°C for 0.5, 1, 2 and 4 h, respectively.

As the intensity of the EPR signal corresponds to the concentration of the iron–sulfur clusters, 16% of increasing and 18% of decreasing in the concentration of the [3Fe–4S] and the [4Fe–4S] cluster were observed compared with that before the incubation, respectively, after the incubation at 80°C for 30 min. Because Bs-Fd has the same number of [3Fe–4S] and [4Fe–4S] cluster in the molecule, the above results show that the increase in concentration of [3Fe–4S] cluster is same as the decrease of [4Fe–4S] cluster. This suggests that the conversion of the [4Fe–4S] cluster took place to form the [3Fe–4S] cluster in Bs-Fd. The EPR signal of the $[3Fe–4S]^{1+}$ did not change in the lineshape by heat treatment, showing that the [3Fe–4S] cluster formed by heat treatment is not distinguishable from the original [3Fe–4S] cluster. Prolonged incubation caused decreasing of the [3Fe–4S] and [4Fe–4S] cluster in the concentration showing that the decomposition of the iron–sulfur clusters had taken place.

The decomposition of iron–sulfur clusters in Bs-Fd is thought to proceed as shown in Eqs. 1

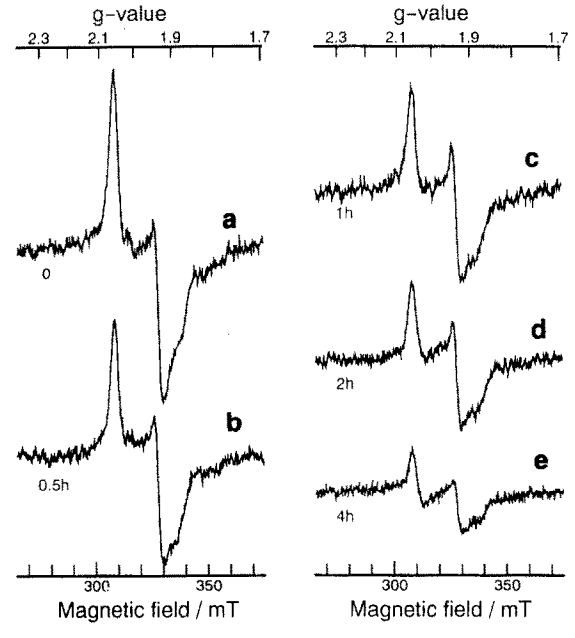


Fig. 6. The EPR spectra of the reduced Bs-Fd (a) before, and after (b) 0.5, (c) 1, (d) 2, and (e) 4 h of heat treatment under aerobic conditions at 80°C. The Bs-Fd was reduced by dithionite after heat treatment for measurement of the EPR spectrum.

and 2 because only EPR signals of $[3Fe\text{–}4S]^{1+}$ and $[4Fe\text{–}4S]^{1+}$ are observed and those of other intermediates are not. The [4Fe–4S] cluster is converted to the [3Fe–4S] cluster at first, and then the [3Fe–4S] cluster formed is decomposed.

$$[4Fe\text{–}4S] \rightarrow [3Fe\text{–}4S] \rightarrow \text{decomposition} \quad (1)$$

The original [3Fe–4S] is bleached directly without any stable intermediates.

$$[3Fe\text{–}4S] \rightarrow \text{decomposition} \quad (2)$$

The electron transfer activity of the heat treated Bs-Fd corresponded to the concentration of the [3Fe–4S] cluster and not to that of the [4Fe–4S] cluster as shown in Table 1. This suggests that the only [3Fe–4S] cluster acts as an active site for the reduction of cyt. *c*. This hypothesis can explain the increasing of the activity by the incubation at 80°C for 30 min, i.e., the conversion of the [4Fe–4S] cluster to the [3Fe–4S] cluster proceeded by heat treatment and the concentration of the [3Fe–4S] cluster as the active site increased with increasing of the activity. The redox properties of the original [3Fe–4S] cluster and the [3Fe–4S] cluster converted from the [4Fe–4S] cluster are thought to be identical because their EPR spectra were not distinguishable.

The [4Fe–4S] cluster is thought not to act as the active site for the electron transfer in the reduction of cyt. *c* as described above. This may be caused by a low redox potential of the [4Fe–4S] cluster in Bs-Fd. It has reported that the redox potential of [4Fe–4S] cluster in *Azotobacter*-type 7Fe ferredoxins is too low to reduce it by dithionite. For Bs-Fd, about 70% of the [4Fe–4S] cluster, not 100%, is reduced by dithionite [10]. The incomplete reduction of the [4Fe–4S] cluster is thought to be caused by low redox potential of the [4Fe–4S] cluster. As the redox potential of NADPH is −320 mV (at pH 7.0), the [4Fe–4S] cluster in Bs-Fd cannot be reduced in the reaction system shown in Scheme 1. The only [3Fe–4S] cluster, therefore, is redox active in the above reaction system to act as the active site.

Acknowledgements

We would like to thank Professor D. Ohmori of Juntendo University for the measurement of EPR spectra. The author (S.A.) thanks Kihara Memorial Yokohama Foundation for the Advancement of Life Sciences for the financial support.

References

[1] M. Bruschi and F. Guerlesquin, FEMS Microbiol. Rev., 54 (1988) 155.
[2] H. Matsubara and K. Saeki, Adv. Inorg. Chem., 38 (1992) 223.
[3] J.J.G. Moura, A.V. Xavier, E.G. Hatchikian and J. LeGall, FEBS Lett., 89 (1978) 177.
[4] F.A. Armstrong, S.J. George, A.J. Thomson and M.G. Yates, FEBS Lett., 107 (1988) 107.
[5] M. Bruschi, E.C. Hatchikian, J. LeGall, J.J.G. Moura and A.V. Xavier, Biochim. Biophys. Acta, 449 (1976) 275.
[6] J.J.G. Moura, J. LeGall and A.V. Xavier, Eur. J. Biochem., 141 (1984) 319.
[7] B. Guigliarelli, P. Bertrand, C. More, P. Papavassiliou, E.C. Hatchikian and J.P. Gayda, Biochim. Biophys. Acta, 810 (1985) 319.
[8] M.K. Johnson, A.J. Thomson, A.J.M. Richards, J. Peterson, A.E. Robinson, R.R. Ramsay and T.P. Singer, J. Biol. Chem., 259 (1984) 2274.
[9] T.A. Kent, J.L. Dreyer, M.C. Kennedy, B.H. Huynh, M.H. Emptage, H. Beinert and E. Münck, Proc. Natl. Acad. Sci. USA, 79 (1982) 1096.
[10] S. Aono, H. Kurita, S. Uno and I. Okura, J. Biochem., 112 (1992) 792.
[11] A. Schenk and M. Aragno, J. Gen. Microbiol., 115 (1979) 333.
[12] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
[13] S. Sato, K. Nakazawa, K. Hon-nami and T. Oshima, Biochim. Biophys. Acta, 668 (1981) 277.
[14] M.F. Perutz and R.H. Raidt, Nature (London), 255 (1975) 256.
[15] J.B. Howard, T. Lorsbach and L. Que, Biochem. Biophys. Res. Commun., 70 (1976) 582.
[16] T. Hase, S. Wakabayashi, H. Matsubara, D. Ohmori and K. Suzuki, FEBS Lett., 91 (1978) 315.
[17] T. Hase, S. Wakabayashi, H. Matsubara, T. Imai, T. Matsumoto and J. Tobari, FEBS Lett., 103 (1979) 224.
[18] S. Aono, S. Nakamura, R. Aono and I. Okura, Biochem. Biophys. Res. Commun., 201 (1994) 938.
[19] D. Schlatter, S. Waldvogel, F. Zubti, F. Suter, W. Portmann and H. Zuber, Biol. Chem. Hoppe-Seyler, 366 (1985) 223.