Biochemistry (Moscow), Vol. 68, No. 2, 2003, pp. 177-181. Translated from Biokhimiya, Vol. 68, No. 2, 2003, pp. 212-217.
Original Russian Text Copyright © 2003 by Solovieva, Tarasov, Perumov.

Main Physicochemical Features of Monofunctional Flavokinase from *Bacillus subtilis*

I. M. Solovieva*, K. V. Tarasov, and D. A. Perumov

Department of Molecular and Radiation Biophysics, Konstantinov St. Petersburg Institute of Nuclear Physics, Russian Academy of Sciences, Gatchina, Leningrad Region, 188350 Russia; fax: (812) 247-9191; E-mail: solovieva@em.uni-frankfurt.de

Received December 13, 2001
Revision received April 8, 2002

Abstract—The main properties of a monofunctional riboflavin kinase from *B. subtilis* have been studied for the first time; the enzyme is responsible for a key reaction in flavin biosynthesis—the ATP-dependent phosphorylation of riboflavin with production of flavin mononucleotide. The active form of the enzyme is a monomer with molecular weight of about 26 kD with a strict specificity for reduced riboflavin. To display its maximum activity, the enzyme needs ATP and Mg^{2+}. During the phosphorylation of riboflavin, Mg^{2+} could be partially replaced by ions of other bivalent metals, the efficiencies of which decreased in the series $Mg^{2+} > Mn^{2+} > Zn^{2+}$, whereas Co^{2+} and Ca^{2+} had inhibiting effects. The flavokinase activity was maximal at pH 8.5 and 52°C. ATP could be partially replaced by other triphosphates, their donor activity decreasing in the series: ATP > dATP > CTP > UTP. The Michaelis constants for riboflavin and ATP were 0.15 and 112 μM, respectively. As compared to riboflavin, a tenfold excess of its analog 7,8-dimethyl-10-(O-methylacetoxime)-isoalloxazine decreased the enzyme activity by 30%. Other analogs of riboflavin failed to markedly affect the enzyme activity.

Key words: *Bacillus subtilis*, flavinogenesis, monofunctional flavokinase, physicochemical properties

The flavinogenesis system of *B. subtilis* includes the biosynthesis of riboflavin and of its active coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Until recently it had been thought that on the structural gene level the flavinogenesis of *B. subtilis* included a riboflavin operon [1, 2] and the gene of bifunctional riboflavin kinase/FAD-synthase *ribC* [3, 4] located at 209 and 148° of the chromosome map, respectively [5]. However, we have recently shown that the *B. subtilis* chromosome has another gene which is directly associated with flavinogenesis, the *ribR* gene encoding the monofunctional flavokinase. This gene is located at 256° and is a constituent of a linkage group of 12 genes which has all features of an operon [6]. The gene *ribR* copy prepared by the polymerase chain reaction (PCR) was subcloned in *Escherichia coli* cells on the pRSET-5d vector under the control of an inducible phage T7 promotor. Corresponding to the gene size of 690 nucleotide pairs, the *ribR* gene produced a polypeptide with the molecular weight of about 26 kD which was a monofunctional flavokinase, the enzyme responsible for only the first step in riboflavin utilization, the riboflavin phosphorylation with production of FMN [7]. There are now data on several enzymes, both monofunctional and bifunctional, which are responsible for similar reactions. The bifunctional enzymes catalyze FMN production from riboflavin and the further conversion of FMN into FAD with the involvement of ATP. These enzymes from various sources have been partially or completely purified. These enzymes include: the monofunctional riboflavin kinase and the monofunctional FAD-synthase (ATP-FMN adenilyl transferase) from rat liver [8], flavokinase from *Neurospora crassa* [9], and bifunctional riboflavin kinases/FAD-synthases from *Corynebacterium ammoniagenes* [10, 11], *E. coli* [11], and *B. subtilis* [3, 12].

In the present work the main physicochemical features of the monofunctional flavokinase from *B. subtilis* were studied. The results seem useful for studies on the flavinogenesis system, in particular, in *B. subtilis* which is not only one of the main objects of molecular biology of procaryotes but is also widely used in modern biotechnology as a producer of various physiologically active compounds.

* To whom correspondence should be addressed.

0006-2979/03/6802-0177$25.00 ©2003 MAIK "Nauka/Interperiodica"

MATERIALS AND METHODS

Reagents. All working solutions were prepared using reagents from Sigma (USA), Serva (Germany), and domestic reagents of no less than special purity qualification. Riboflavin and FMN were from Serva and [γ-^{33}P]ATP and [2-^{14}C]riboflavin were from Amersham (Great Britain). 6,7-Dimethyl-10-(O-methylacetoxime)-isoalloxazine was prepared by the method of Fall and Petering [13].

Preparation of cell extracts. Cell extract from *E. coli* enriched with the protein of our interest was prepared by the procedure recommended by Novagen (England). The cell extracts were prepared with an UDN-1 ultrasonic disintegrator at the frequency of 44 kHz at 0°C. The sonication was performed for 5 min with intervals for cooling. The cell fragments were separated by centrifugation at 10,000*g*.

Protein purification. The protein of the cell extracts was purified by affinity chromatography on Ni^{2+}-agarose as recommended by Novagen. The cell extract was incubated with agarose at 4°C with slight rocking. The unbound protein was separated by centrifugation, and the agarose was washed thrice with 50 mM Na_2HPO_4 buffer with 300 mM NaCl (pH 8) containing 20 mM imidazole. The protein was eluted with buffer containing 200 mM imidazole. After dialysis, the sample was placed onto a Mono S HR 5/5 cation-exchange column (Pharmacia, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 7.7) and 0.1 mM EDTA. The bound protein was washed with 20 ml of this buffer at the flow rate of 0.5 ml/min and eluted with 30 ml of a linear NaCl gradient from 0 to 500 mM in the same buffer. The RibR protein was eluted with 200 mM NaCl. Fractions containing the recombinant protein were dialyzed and concentrated with a Microcon YM-10 microconcentrator (Amicon, Holland).

The protein concentration in the extracts was determined as described in [14] with reagents from Bio-Rad (USA).

Determination of protein molecular weight by gel filtration. A column (1 × 30 cm) with Sephacryl 300 was equilibrated with three volumes of 50 mM Tris-HCl buffer (pH 7.7) containing 0.1 mM EDTA. The standard proteins and those to be determined were placed onto the column at the flow rate of 3.6 ml/h. For the calibration curve the following proteins were used as standards: ferritin (440 kD), BSA dimer (132 kD), BSA monomer (67 kD), ovalbumin (45 kD), chymotrypsinogen (25 kD), and cytochrome *c* (14 kD). The free volume of the column was determined with Dextran Blue 2000. The molecular weight of flavokinase was determined from the dependence curve of molecular weights of the standard proteins on their $K_v = (V_e - V)/(V_o - V)$, where V_e, V_o, and V were the volume eluted, the total volume, and the free volume of the column, respectively.

Determination of the flavokinase activity. The flavokinase activity was determined as described in [15]. The reaction mixture (final volume 0.6 ml) contained 100 mM Tris-HCl buffer (pH 8.0), riboflavin, and ATP in the concentration depending on the experiment purpose, 10 mM $MgCl_2$, and 20 mM NaF. The standard solution of riboflavin was supplemented with [2-^{14}C]riboflavin with the specific radioactivity of 800-1000 cpm/nmol. Immediately before the addition of extract, the reaction mixture was supplemented with $Na_2S_2O_4$ to the concentration of 10 mM to provide the riboflavin reduction, and the reaction was performed under a mineral oil (Sigma) layer to prevent riboflavin reoxidation. The protein content in the reaction mixture was 0.1-0.03 mg/ml. The reaction was performed at 37°C and stopped by heating at 100°C for 5 min. Aliquots (10-20 μl) were taken at different times and placed onto Whatman 3 MM chromatographic paper. Riboflavin (R_f = 0.46) and FMN (R_f = 0.85) were separated by ascending chromatography in a water–isoamyl alcohol system [16]. Spots of riboflavin and FMN were located by UV irradiation, cut out, and their radioactivity was determined in a Zh-107 scintillator with an SL 30 spectrometer (Intertechnique, France).

The amount of the enzyme producing 1 nmol FMN per 1 min at 37°C was taken as the unit of the enzyme specific activity.

RESULTS

The PCR-copy of the *ribR* gene was prepared with chromosomal DNA using primers which contained the restriction sites for the gene cloning into the pET-28a plasmid (Novagen). The cloning with this vector did not change the N-terminal of the future peptide and introduced six additional histidine residues on the C-terminal of the protein that allowed us to use Ni^{2+}-agarose for its purification. The *ribR* gene was put under the control of a promotor induced by isopropyl-β-*o*-thiogalactopyranozide (IPTG). The recombinant protein was purified from the extract of *E. coli* cells after induction for 4 h using Ni^{2+}-agarose and by a subsequent cation-exchange chromatography on a MONO-S column. This resulted in electrophoretically pure protein (Fig. 1). The specific activity of purified flavokinase in the preparation was 700 units, whereas the baseline level of intrinsic flavokinase/FAD-synthase of the strain BL21 (DE3) cells before the RibR protein induction was no more than 0.1 unit. The reaction rate of the enzyme linearly depended on the incubation time during 30 min at 37°C, and thus, the determination of the main features was correct.

Use of reduced riboflavin. The flavokinase under study could use only reduced riboflavin. When oxidized riboflavin was used as a substrate no FMN was detected at the riboflavin concentration of 50 μM and at the incubation time from 30 min to 2 h. In the control experiment

Table 1. Effects of different metal ions on flavokinase activity

Metal	Production of FMN after 10 min, μM	
	Concentration of ions	
	1 mM	5 mM
$MgCl_2 \cdot 6H_2O$	27.58	50.66
$ZnCl_2$	12.99	21.17
$MnCl_2 \cdot 4H_2O$	32.27	30.88
$CuCl_2 \cdot 2H_2O$	0	0
$CaCl_2$	0.63	0
Without additions	7.15	

Note: The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 20 mM NaF, 10 mM $Na_2S_2O_4$, 50 μM riboflavin, 1 mM ATP, and the enzyme. The reaction was performed at 37°C.

Table 2. Effects of riboflavin analogs on flavokinase activity

Riboflavin analog	Production of FMN after 10 min, μM	Inhibition of the enzyme activity, %
Riboflavin, without analog	45.6	—
Roseoflavin, 400 μM	41.9	8
7,8-Dimethyl-10-(O-methylacetoxime)-isoalloxazine, 500 μM	30.3	34
7,8-Dimethyl-10-(2′-hydroxyethyl)-isoalloxazine, 500 μM	41	10
Lumiflavin, 500 μM	43.7	4
Lumichrome, 420 μM	41.5	9

Note: The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 20 mM NaF, 10 mM $Na_2S_2O_4$, 50 μM riboflavin, the riboflavin analog in the concentration indicated, 1 mM ATP, and the enzyme. The reaction was performed at 37°C.

with reduced riboflavin the specific activity of the enzyme was ~600 units.

Effects of temperature and pH on flavokinase activity. Flavokinase activity was determined in a standard reaction mixture containing 50 μM riboflavin, 1 mM ATP, and the enzyme, and the optimum enzyme activity was recorded at 52°C and pH 8.5.

Effects of bivalent cations of different metals. Effects of Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} on the activity of RibR-kinase were studied. The resulting production of FMN after 10 min incubation is presented in Table 1. For the maximum enzyme activity Mg^{2+} was required, Mn^{2+} and Zn^{2+} were less effective, and Co^{2+} and Ca^{2+} displayed inhibiting effects.

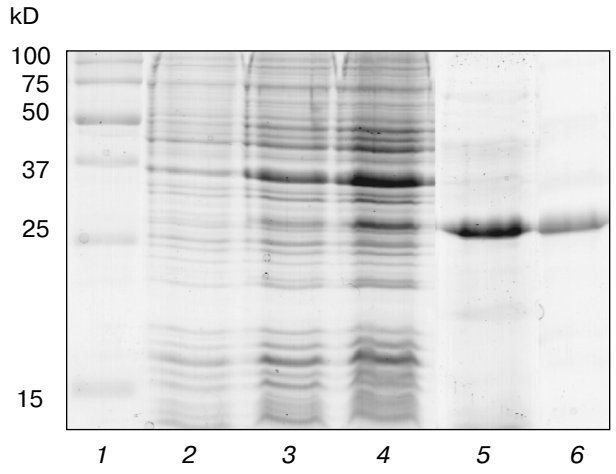

Fig. 1. Purification of RibR protein. SDS-polyacrylamide gel: the standard proteins for determination of the molecular weight (*1*), *E. coli* BL21 (DE3) cell extract containing the gene *ribR* copy: before induction (lane *2*), and 2 and 4 h after the induction with IPTG (lanes *3* and *4*, respectively); the protein fraction after purification on Ni^{2+}-agarose (lane *5*); the protein fraction after cation-exchange chromatography (lane *6*).

Effects of riboflavin analogs on flavokinase activity. We studied effects on the flavokinase activity of some riboflavin analogs, such as roseoflavin (RSF, 8-dimethylamino-riboflavin), 7,8-dimethyl-10-(O-methylacetoxime)-isoalloxazine (MO), lumiflavin (LF, 7,8,10-trimethylisoalloxazine), 7,8-dimethyl-10-(2′-hydroxyethyl)-isoalloxazine (riboflavin E), and lumichrome (LC, 7,8-dimethylalloxazine). These analogs were chosen based on the following reasons. RSF is widely used for selection of *B. subtilis* strain producers of riboflavin; its regulatory activity with respect to the riboflavin operon has been shown [17]. The *ribR* gene was found in mutants resistant to MO [6, 18]; and LF, LC, and riboflavin E are products of riboflavin photolysis [17, 19]. The experiments were performed in the presence of the 8-10-fold increased concentrations of the analogs in the reaction mixture over the concentration of riboflavin. The results are presented in Table 2. Only MO had a noticeable effect on the enzyme activity.

Phosphate group donors. Was it possible to use other sources of phosphate than ATP for riboflavin phosphory-

Table 3. Use of various donors of phosphate group for the flavokinase reaction

Substrate	Enzyme activity, %	Substrate	Enzyme activity, %
ATP	100	dATP	54
GTP	1	dGTP	0
CTP	50	dCTP	0
TTP	2	dTTP	0
UTP	31		
Glucose-6-phosphate	1.6		

Note: The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 20 mM NaF, 10 mM $Na_2S_2O_4$, 50 μM riboflavin, the enzyme, 1 mM substrate (triphosphates, deoxytriphosphates, or glucose-6-phosphate). The reaction was performed at 37°C.

lation? As alternative sources of phosphate the following substances were tested: GTP, CTP, TTP, dATP, dCTP, dGTP, dTTP, glucose-6-phosphate, polyphosphates. The results in the presence of triphosphates, deoxytriphosphates, and glucose-6-phosphate are presented in Table 3. In addition to ATP, other triphosphates also could be donors of phosphate for the riboflavin phosphorylation, and the donor activity of dATP and CTP differed from that of ATP not more than twofold. GTP, dGTP, dCTP, dTTP, and glucose-6-phosphate had no donor activity.

Metaphosphate as a source of phosphate for the riboflavin phosphorylation was found to react with riboflavin with FMN yield near that in the case when ATP was used [15]. In the present work standard polyphosphates from Sigma were used: Phosphate glass, Trisodium trimetaphosphate ($Na_3P_3O_9$), Tripolyphosphate ($Na_5P_8O_{18}$). The concentration of the polyphosphate solutions used was 10 mg/ml. The reaction was performed under standard conditions within 2 h. The control reaction with ATP was completed, whereas no sample with polyphosphates gave a spot of FMN in the chromatogram.

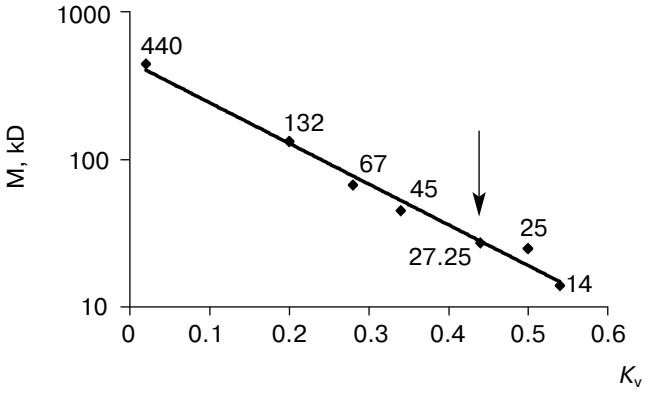

Fig. 2. Determination of molecular weight of native RibR protein by gel filtration. The dependence curve of the protein molecular weights (logarithmic scale) on their K_v (relative volume of elution) is presented. For flavokinase K_v was 0.44 (the flavokinase position is indicated with the arrow).

Determination of molecular weight of the native flavokinase protein. The molecular weight of the native flavokinase was determined by gel filtration on Sephacryl 300. The K_v value for flavokinase was found to be 0.4, corresponding to the molecular weight of 27.25 kD (Fig. 2). Hence, the active form of the enzyme is a monomer.

Determination of Michaelis constants (K_m) for each substrate. Values of K_m were determined for ATP and riboflavin. The reaction was performed by the standard procedure during 5 min, the time when the time-dependence of the FMN production was linear, as had been previously found. The K_m^{ATP} value was determined at the fixed concentration of riboflavin (RF) of 25 μM, the K_m^{RF} value was determined at the fixed concentration of ATP of 5 mM. The results were linearized by the Lineweaver–Burk method. For riboflavin the K_m value was 0.180 μM and for ATP K_m was 112 μM.

DISCUSSION

The main features of the studied protein suggest it to be a flavokinase, and both mono- and bifunctional flavokinases are known. However, there are some differences. Thus, the bifunctional flavokinase/FAD-synthase from *Corynebacterium ammoniagenes* had an interesting specific feature: along with ATP it could use $Na_3P_3O_9$ as a donor of phosphate [15]. However, notwithstanding a high homology degree between the RibR protein of *B. subtilis* and the C-terminal of the bifunctional flavokinase/FAD-synthase from *C. ammoniagenes* (up to 45% identity in the sequence of 120 amino acids), such a change of the phosphate group donor for polyphosphates was impossible for the RibR protein.

Among the riboflavin analogs studied in the present work only MO had an effect on the enzyme activity. The resistance genotype of *B. subtilis* to this analog was earlier shown to have a complicated multiple character [19]; therefore, it was reasonable to find resistance mutations in the structural region of the *ribR* gene. As to the lack of effect of other analogs, difference was found between the features of the bifunctional and monofunctional enzymes from *B. subtilis*: the bifunctional enzyme was sensitive to both LF and riboflavin E, and at the same substrate/analog ratio (1 : 10) the kinase activity of the bifunctional enzyme was inhibited by 34 and 38%, respectively [20].

Values of K_m of the flavokinase reaction of the bifunctional flavokinase/FAD-synthase (0.05-0.1 μM for

riboflavin and 6.5 μM for ATP [20]) are significantly lower than the corresponding values of the monofunctional protein. Consequently, the RibR protein starts functioning at higher intracellular concentrations of riboflavin when the activity of the bifunctional enzyme begins to decrease for some reason. Note, that the monofunctional flavokinase activity was first found in MO-resistant mutants with inhibited flavokinase activity of the *ribC* gene [6, 18]. But even under these conditions the amount of the RibR protein in the cell was small, and we failed in its detection before the amplification of the *ribR* gene. Consequently, the *ribR* gene obviously played a satellite function in the flavinogenesis itself of *B. subtilis*. The chromosome encirclement of the *ribR* gene presented by the region of 12 genes (256° in the chromosome map of *B. subtilis*) has some specific features suggesting that the *ribR* gene is a part of a heterogeneous operon. When is this operon "switched on" and what is its role in the total metabolism picture? To answer these questions is the task of future work.

This work was supported by the Russian Foundation for Basic Research (project No. 00-04-48868).

REFERENCES

1. Bresler, S. E., Cherepenko, E. I., Chernik, T. P., Kalinin, V. L., and Perumov, D. A. (1970) *Genetika*, **6**, 116-124.
2. Mironov, V. N., Kraev, A. S., Chikindas, M. L., Chernov, B. K., Stepanov, A. I., and Skryabin, K. G. (1994) *Mol. Gen. Genet.*, **242**, 201-208.
3. Gusarov, I. I., Kreneva, R. A., Iomantas, Yu. A., Kolibaba, L. G., Polanuer, B. M., Kozlov, Yu. I., and Perumov, D. A. (1997) *Mol. Biol. (Moscow)*, **31**, 825-830.
4. Mack, M., van Loon, A. P. G. M., and Hohman, H. P. (1998) *J. Bacteriol.*, **180**, 950-955.
5. Kreneva, R. A., and Perumov, D. A. (1990) *Mol. Gen. Genet.*, **222**, 467-469.
6. Kreneva, R. A., and Perumov, D. A. (1996) *Genetika*, **10**, 1-8.
7. Solovieva, I. M., Kreneva, R. A., Leak, D. L., and Perumov, D. A. (1999) *Microbiology*, **145**, 67-73.
8. Oka, M., and McCormick, D. B. (1987) *J. Biol. Chem.*, **262**, 7418-7422.
9. Rajeswari, S. R., Jonnalagadda, V. S., and Jonnalagadda, S. (1999) *Ind. J. Biochem. Biophys.*, **36**, 137-142.
10. Manstein, D. J., and Pai, E. F. (1986) *J. Biol. Chem.*, **261**, 16169-16173.
11. Nakagawa, S., Igarashi, A., Ohta, T., Hagihara, T., Fujio, T., and Aisaka, K. (1995) *Biosci. Biotech. Biochem.*, **59**, 694-702.
12. Coquard, D., Huecas, M., Ott, M., Vandijl, J. M., Vanloon, A. P., and Hohmann, H. P. (1997) *Mol. Gen. Genet.*, **254**, 82-84.
13. Fall, H. H., and Petering, H. G. (1956) *J. Am. Chem. Soc.*, **75**, 377-380.
14. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
15. Hagihara, T., Fujio, T., and Aisaka, K. (1995) *Appl. Microbiol. Biotechnol.*, **42**, 724-729.
16. Hais, I. M., and Macek, K. (eds.) (1962) in *Paper Chromatography* [Russian translation], Izd-vo Inostrannoi Literatury, Moscow, pp. 610-613.
17. Stepanov, A. I., Kukanova, A. Yu., Glazunov, E. A., and Zhdanov, V. G. (1977) *Genetika*, **13**, 490-495.
18. Solovieva, I. M., Iomantas, Yu. A.-V., Kreneva, R. A., Kozlov, Yu. I., and Perumov, D. A. (1997) *Genetika*, **33**, 739-743.
19. Kreneva, R. A., and Perumov, D. A. (1994) *Biotekhnologiya*, **3**, 6-10.
20. Kearney, E. B., Goldenberg, J., Lipsick, J., and Perl, M. (1979) *J. Biol. Chem.*, **254**, 9551-9557.