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A CONTINUOUS FLUOROMETRIC ASSAY FOR FLAVOKINASE

PROPERTIES OF FLAVOKINASE FROM *PEPTOSTREPTOCOCCUS ELSDENII*

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Summary

A continuous fluorometric assay that utilizes apoflavodoxin as a trapping agent for riboflavin 5'-phosphate (FMN) has been developed for flavokinase (ATP:riboflavin 5'-phosphotransferase, EC 2.7.1.26). Use of this assay is illustrated in a procedure for the partial purification of flavokinase from the strict anaerobe *Peptostreptococcus elsdenii.* The purified enzyme catalyzed the formation of 8.3 nmol FMN · min^{-1} · mg^{-1} at 37°C and had apparent K_m values for riboflavin and ATP of 10 and 4.7 μM, respectively. ATP could be replaced by ADP (22% of the rate observed with ATP) but not by GTP. The enzyme also phosphorylated 5-deaza- and 8-bromoriboflavin with activities of 15 and 70%, respectively, of that with riboflavin; it was inactive with iso riboflavin and deoxyriboflavin.

Introduction

The enzyme flavokinase (ATP:riboflavin 5'-phosphotransferase, EC 2.7.1.26) catalyses the phosphorylation of riboflavin to riboflavin 5'-phosphate (FMN) [1], and has been detected in extracts of rat liver [2], plant leaves [3—6], yeasts [1] and bacteria [7—9]. It has been extensively purified from rat liver by affinity chromatography [10] but only partially purified from other sources. Most flavokinases examined to date utilize ATP as the specific phosphate donor,

$$\text{Riboflavin} + \text{ATP} \rightarrow \text{riboflavin 5'-phosphate} + \text{ADP} \tag{1}$$

but ADP is also effective in certain cases [1,5] and flavokinase from *Rhizopus* spp is specific for GTP [11].

This enzymic reaction is of interest because, in contrast with chemical methods which lead to a number of phosphorylated flavin products that are difficult to separate [13,14,23], the enzyme phosphorylates riboflavin only at the 5′ position. Enzymic synthesis of FMN and FMN analogues could circumvent uncertainties associated with the use of commercial samples of FMN, and chemically synthesized analogues, several of which have been found to be very impure [13,14].

Unfortunately the course of the enzymic reaction cannot be followed directly by spectrophotometry or fluorometry because the spectroscopic properties of riboflavin and FMN are the same. The published assay for flavokinase involves a fixed time of incubation (usually 1 h at 37°C) followed by extraction of the reaction mixture with organic solvents and spectrophotometric measurement of the distribution of riboflavin and FMN in the aqueous and organic phases [15,16]. Consequently it is time consuming, and not very sensitive. We have developed a more convenient assay based on the observation that the apoenzymes of certain flavodoxins show an unusually high specificity for FMN; they rapidly bind one molecule of FMN, causing more than 99% quenching of the flavin fluorescence, but they do not react with riboflavin [17]. Addition of such apoflavodoxins to the assay for flavokinase therefore allows the phosphorylation of riboflavin to be followed continuously by fluorometry. This paper describes the assay and its use in the partial purification and characterization of flavokinase from *Peptostreptococcus elsdenii*.

Materials and Methods

Procedures for the growth of *P. elsdenii* [18], the purification of flavodoxin [18] and the preparation of apoflavodoxin [14] have been described. Flavodoxin was also obtained during the course of the flavokinase purification described below. Concentrations of apoflavodoxin were determined by fluorometric titration with standard FMN [14]. Riboflavin was obtained from Sigma and determined spectrophotometrically with the use of an extinction coefficient of 12 500 $M^{-1} \cdot cm^{-1}$ at 445 nm [19]. Protein was determined by the biuret method [20] and electrophoresis was carried out in 8% polyacrylamide gels [21].

Assay of flavokinase. Incubation mixtures for the assay of flavokinase were modified from that of McCormick [16] and contained in a final volume of 2 ml at 37°C: 50 μM riboflavin, 0.1 mM ATP, 8 mM $MgCl_2$, 50 μM $ZnSO_4$, 25 mM potassium phosphate buffer, pH 7.2, 10 μM apoflavodoxin and flavokinase. Reactions were begun by addition of ATP. Fluorescence change was followed with an Eppendorf front face fluorometer (1101 M) equipped with a Hg lamp, filters in the excitation (436 nm) and emission (Schott filter GG 495) light beams, and a back-off device that allowed removal of 90% of the emitted light and expansion of the remainder over the full scale of the instrument. The expansion device permitted initial rates to be determined with low concentrations of apoflavodoxin. The experiments of Fig. 1 were carried out in a Hitachi-Perkin Elmer spectrofluorometer MPF-2A with the emission and excitation monochrometers set at 530 and 445 nm, respectively. Due to self-absorption at the high riboflavin concentration required for the assay, and with exciting light at the wavelength that proved convenient in the filter fluorometer, the observed fluorescence was not linear with flavin concentration. However, since the total flavin concentration did not change during the assay, and the absorbance change due to the binding of FMN to apoflavodoxin was small [17], self-absorption remained constant, and the observed changes in the fluorescence were linear with the concentration of FMN synthesized. Instrumental limitations made it necessary to make routine measurements at a riboflavin concentration (50 μM) that was closer than desirable to the K_m of riboflavin (10 μM) for *P. elsdenii* flavokinase.

Purification of flavokinase. Flavokinase was partially purified from extracts of *P. elsdenii* grown in iron-poor medium [18] by a procedure similar to that described by McCormick [16] for the enzyme in rat liver. Frozen cell paste, 50 g, was thawed out, suspended in 100 ml 10 mM phosphate buffer, pH 7.2, and the cells then broken by sonification (M.S.E. sonifier, 3 min at 4°C). The suspension of broken cells was centrifuged (36 000 × *g*, 60 min), and the proteins in the supernatant fractionated by addition of solid $(NH_4)_2SO_4$. The precipitate that formed between 40 and 55% saturation with ammonium sulphate was collected by centrifugation (30 000 × *g*, 15 min), dissolved in about 20 ml 10 mM phosphate buffer, pH 7.2, and dialysed for 10 h versus the same buffer. The brownish solution was then applied to a column of diethylaminoethylcellulose (DEAE-cellulose, 26 × 3 cm diameter) equilibrated with 10 mM phosphate buffer, pH 7.2, and the column subsequently developed with a linear salt gradient made by continuously diluting 500 ml 10 mM phosphate buffer, pH 7.2, with 500 ml 0.2 M phosphate buffer, pH 7.2. Fractions from the column that contained flavokinase activity were combined and the enzyme precipitated by adding $(NH_4)_2SO_4$ to 60% saturation. The precipitate was collected by centrifugation, suspended in 10 mM phosphate buffer, pH 7.2, dialysed versus the same buffer, and the dialysed solution was then centrifuged to remove a small amount of insoluble material. The solution of enzyme (18 ml) was then divided into three and each portion chromatographed by gel filtration in a column of Sephadex G-150 (90 × 2.5 cm diameter) equilibrated with 10 mM phosphate buffer, pH 7.2. Fractions that contained flavokinase with the highest specific activity were combined, concentrated by precipitation with ammonium sulphate and subsequently dialysed as described above.

No losses in activity were observed after storage of this preparation for several weeks in frozen solution at −20°C. Activity losses did occur, however, during repeated freezing and thawing. Therefore the concentrated enzyme was divided into portions of about 1 ml before freezing. Portions of enzyme were thawed as required and subsequently stored at 4°C.

Flavodoxin in the extracts could be recovered and further purified for use in its apoenzyme form in the assay for flavokinase. Most of the flavodoxin remained in the supernatant after precipitation of flavokinase with 55% saturated $(NH_4)_2SO_4$. It could be recovered by adsorption to DEAE-cellulose that had been equilibrated with ammonium sulphate [22]. A small part of the total flavodoxin in the extracts was precipitated with flavokinase, but the two proteins were separated during the subsequent chromatographic step; flavodoxin remained on the column after removal of the flavokinase. Flavodoxin-containing fractions from these two steps of the purification procedure were combined and the protein further purified by using the chromatographic and salt fractionation procedures described previously [18].

Results and Discussion

Flavokinase assay

In contrast with the procedure commonly used to assay flavokinase activity [16], the fluorometric assay described in Materials and Methods allowed the time course of the reaction to be followed continuously (Fig. 1) and permitted an accurate determination of the initial rate. No fluorescence changes occurred when either ATP, apoflavodoxin or enzyme were omitted from the assay mixture (Table I), and in the complete system the rate of fluorescence decrease was linear with the concentration of enzyme. Use of less apoflavodoxin than riboflavin in the assay limited the extent of fluorescence decrease but did not affect the rate (Fig. 1); hence, after expansion of the highest 10% of the riboflavin fluorescence, 5 μM apoflavodoxin was sufficient for initial rate measurements. Even this concentration of apoenzyme might appear to be costly. However, *P. elsdenii* flavodoxin is readily prepared in large quantities [18], and if necessary, it can be recovered from the assay mixture by ion-exchange chromatography.

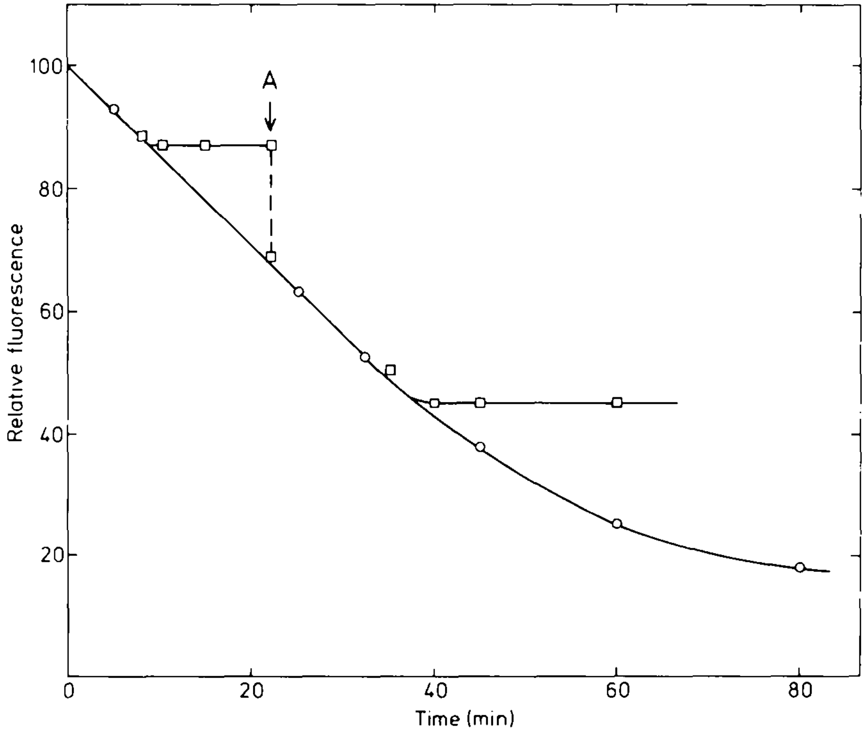

Fig. 1. Time course of fluorescence decrease during FMN synthesis. Reaction mixtures contained in a final volume of 2 ml at 37°C: 25 mM potassium phosphate buffer, pH 7.2, 0.1 mM riboflavin, 0.1 mM ATP, 8 mM $MgCl_2$, 50 μM $ZnSO_4$, 0.3 mg flavokinase, and apoflavodoxin. ○———○, the apoflavodoxin concentration was 0.126 mM; □———□, the initial apoflavodoxin concentration was 14 μM; a second addition of apoflavodoxin, 42 μM, was made after 22 min (point A).

TABLE I

FLAVOKINASE ASSAY: COMPONENT STUDY

Experimental conditions were as described in Materials and Methods. The final concentration of ADP was 0.1 mM.

Component omitted	Rate of fluorescence decrease (arbitrary units/min)
None	6
Flavokinase	0
Apoflavodoxin	0
ATP	0
ATP (ADP added)	1.3
$MgCl_2$	5.8
$ZnSO_4$	6
$MgCl_2$, $ZnSO_4$	0

The K_m value for riboflavin has been reported to be about 10 μM for several flavokinases [5,6,11,16], and it is therefore necessary to use concentrations of flavin that are much higher than is usual in fluorometry. However, for reasons noted in Materials and Methods, the observed fluorescence changes were linear with the concentration of FMN formed in the assay.

Purification and properties of P. elsdenii flavokinase

The assay described above was used in the purification of flavokinase from *P. elsdenii*. This strictly anaerobic bacterium is known to contain high levels of flavin [24], and its content of FMN is enhanced after growth in iron-poor medium (Wassink, J.H., Dekker, I., De Bont, A. and Mayhew, S.G., unpublished). We were therefore not too surprised to find that the organism contains rather high levels of flavokinase. The purification procedure that we have used for the enzyme led to a 17-fold purification with an 11% yield of activity (Table II)). The final preparation showed four major bands of protein after electrophoresis in polyacrylamide gel, and a similar number of minor bands; it was therefore still impure. Nevertheless, its specific activity (8.3 nmol FMN formed/min per mg protein at 37°C) was nearly half of that of flavokinase highly purified from rat liver extracts by affinity chromatography (19.3 nmol FMN $\cdot$ min^{-1} $\cdot$ mg^{-1}; ref. 10). The preparation was free of phosphatases that hydrolyse FMN to riboflavin and phosphate ion and which form a persistent contaminant of the rat liver enzyme [16]; addition of apoflavodoxin to incuba-

TABLE II

PURIFICATION OF FLAVOKINASE

The starting material was 50 g of frozen cell paste of *P. elsdenii*

Purification step	Volume (ml)	Total activity (units)	Specific activity (units/mg)
Crude extract	150	1748	0.5
$(NH_4)_2SO_4$ fractionation 40—55% saturation	20	1180	0.6
DEAE-cellulose chromatography	18	720	1.7
Sephadex gel filtration	6	198	8.3

tion mixtures after 40 min of incubation caused an immediate decrease in fluorescence to that of a control reaction in which apoflavodoxin was present from the beginning of the incubation period. No changes in the fluorescence occurred in the absence of apoflavodoxin, indicating that the FMN formed was not further metabolized to FAD, as has recently been observed with a flavokinase preparation from *Brevibacterium ammoniagenes* [12].

When the enzyme reaction was allowed to proceed to completion in the presence of an excess of apoflavodoxin, the final fluorescence attained was 4% of the fluorescence at the beginning of the reaction, indicating either incomplete conversion of riboflavin to FMN, or the presence of flavin contamination [13,14]. The possibility that the enzyme reaction gives rise to phosphorylated products other than riboflavin 5'-phosphate was excluded by carrying out an incubation in the absence of apoflavodoxin, and then separating phosphorylated flavin from other components of the mixture by chromatography on DEAE-cellulose [25]. The fluorescence of this purified sample was quenched by 99.4% during a subsequent titration with apoflavodoxin, showing that it was free of other phosphorylated flavins, such as riboflavin 4'-phosphate which is known to co-chromatograph with FMN [14,23].

A number of properties of the purified enzyme were determined using the assay with apoflavodoxin. The enzyme was active between at least pH 5 and pH 8.5, but showed a broad optimum around pH 7. The relative activities at pH 7.2 and 20, 25, 37 and 45°C were 0.22, 0.46, 1 and 1.35, respectively. The K_m for riboflavin was found to be 10 μM and is therefore similar to those of flavokinases from other sources [5,6,11,16]. An apparent K_m of 4.7 μM was determined for ATP, a value that is considerably lower than has been reported for the rat liver enzyme (0.2 mM [2]). The *P. elsdenii* enzyme also utilized ADP as phosphate donor in the reaction, but the rate with ADP was only 22% of that with ATP (Table I). No activity was observed with GTP.

When a number of riboflavin analogues were substituted for riboflavin in the standard assay, but with appropriate changes in the fluorescence excitation and emission wavelengths, the following relative activities were observed: riboflavin 100; deoxyriboflavin, 0; 5-deazariboflavin, 15; 8-bromoriboflavin, 79; and isoriboflavin, 0. These results suggest that the specificity of *P. elsdenii* flavokinase for flavin substrates is similar to that of the enzyme from rat liver [16]. Consequently, and as discussed by Scola-Nagelschneider and Hemmerich [23], it is not as broadly useful in chemical methods for the phosphorylation of riboflavin derivatives. Since enzymic phosphorylation leads only to 5'-phosphorylated products it is, however, the preferred method for the phosphorylation of flavin substrates that are available in only small quantities, such as those containing radiolabel. *P. elsdenii* appears to be a more suitable source of enzyme for this purpose than either rat liver or yeast.

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