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Plant flavokinase

Affinity-chromatographic procedure for the purification of the enzyme from mung-bean (*Phaseolus aureus*) seeds and conformational changes on its interaction with orthophosphate

J. SOBHANADITYA and N. APPAJI RAO*
Department of Biochemistry, Indian Institute of Science, Bangalore-560 012, India.

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Flavokinase was purified, for the first time from a plant source [mung bean (*Phaseolus aureus*)] by affinity chromatography in the presence of orthophosphate and by using C-8 ATP–agarose (ATP linked through the C-8 position to beaded agarose), Cibacron Blue and riboflavin–Sepharoses. An altered substrates-saturation pattern was observed in the presence of K_2HPO_4. The conformational changes of the enzyme in the presence of K_2HPO_4 were monitored by fluorescence spectroscopy. These results highlight the regulatory nature of this enzyme.

Homoeostasis of flavin coenzymes is achieved by an intricate interplay of the different enzymes in the pathway responding to regulators, such as AMP (Ravindranath & Appaji Rao, 1972; Balakrishnan *et al.*, 1977; Reddy *et al.*, 1979, 1980) and by the altered susceptibility of holo- and apo-flavoproteins to proteolytic digestion (Astrachan *et al.*, 1957; Mitsuda, 1966). Attempts at demonstrating the regulatory features of the first enzyme of the pathway for the biosynthesis of the flavin coenzymes, namely flavokinase (riboflavin kinase or ATP:riboflavin 5'-phosphotransferase, EC 2.7.1.26) were seriously hampered by the unavailability of the enzyme in a homogeneous form, although the enzyme activity was first demonstrated in plants (Giri *et al.*, 1957, 1958; Mitsuda *et al.*, 1963), animals (McCormick, 1961; Sadasivan & Shanmughasundaram, 1966) and micro-organisms (Kearney & Englard, 1951; Oya, 1968; Spencer *et al.*, 1976; Kashchenko & Shavlovskii, 1976; Mayhew & Wassink, 1977) more than 20 years ago. The enzyme from rat liver was recently purified to near homogeneity, and its general properties were reported (Merrill & McCormick, 1980). Here we report the isolation of the enzyme from mung-bean (*Phaseolus aureus*) seeds by a three-step affinity-chromatographic procedure. A unique feature of this purification is the absolute requirement of orthophosphate for the binding of the enzyme to the affinity matrices and the elution of the enzyme with water. The altered substrate-saturation pattern in the presence of K_2HPO_4 suggested that the enzyme could be a regulatory protein.

* To whom correspondence and requests for reprints should be addressed.

Materials and methods

Riboflavin, ATP, FMN, FAD, C-8 ATP–agarose (ATP linked through the C-8 position to beaded agarose) and 6-aminohexyl-Sepharose were purchased from Sigma, St. Louis, MO, U.S.A. All other chemicals used were of analytical-reagent grade. Mung-bean (*Phaseolus aureus*) seeds were purchased from the local market.

C-8 ATP–agarose was kindly given by Dr. T. Satyanarayana, Moffett Field, CA, U.S.A., and the Ultrogel AcA 34 by LKB, Bromma, Sweden.

Preparation of riboflavin–Sepharose

To a solution containing riboflavin (300mg) adjusted to pH 11.0 by the addition of 4M-NaOH was added 4g of CNBr (2g of CNBr/ml of acetonitrile). The pH of the solution was maintained at about 10 by the addition of 4M-NaOH. When the pH of the solution had stabilized for 10min, 6-aminohexyl-Sepharose (packed vol. 15ml) was added to the solution and stirred overnight at 4°C. The affinity gel, after packing into a column (1.5cm × 8cm), was washed with 0.8M-Na_2CO_3/$NaHCO_3$ buffer, pH 10.0 (500ml), followed by 1.0M-sodium acetate buffer, pH 4.0 (500ml), 3.0M-KCl (500ml), water (1 litre) and was equilibrated with 1.0M-potassium phosphate buffer, pH 7.4 (hereafter referred to as, 'buffer A' (500ml). The column was stored at 4°C. The affinity column after use was regenerated by washing it with KCl (500ml, 3.0M), riboflavin (20ml, 1mM) and finally equilibrated with buffer A. This method is a modification of the one described by Kristiansen *et al.* (1969).

Enzyme assays

Flavokinase. The reaction mixture (1ml) con-

tained Tris/HCl buffer, pH 8.5 (200 μmol), riboflavin (0.1 μmol), ATP (1 μmol), $MgSO_4$ (1 μmol), NaF (500 μmol) and enzyme. The reaction mixture was incubated at 37°C for 15–16 min, depending on the stage of the purification of the enzyme. The reaction was stopped by the addition of 0.1 ml of 100% (w/v) trichloroacetic acid and heated in a boiling-water bath (98°C) for 5 min. The reaction mixture was neutralized by the addition of 0.3 ml of 4.0 M-K_2HPO_4. The FMN formed was estimated by partitioning it between water-saturated benzyl alcohol and water as described by Burch *et al.* (1948). One unit of enzyme activity is defined as the amount of enzyme required to form 1 nmol of FMN/min at 37°C and at pH 8.5.

FAD synthetase. The reaction mixture (1 ml) contained FMN (0.1 μmol), ATP (1 μmol), $MgSO_4$ (1 μmol), Tris/HCl buffer, pH 8.5 (200 μmol), NaF (500 μmol) and enzyme. The reaction mixture was incubated at 37°C for 60 min, and the reaction was stopped by the addition of 0.1 ml of 100% trichloroacetic acid and immediately neutralized with 0.3 ml of K_2HPO_4 (4.0 M). FAD was determined by measuring the fluorescence (Bessey *et al.*, 1949) after chromatographic separation (Giri & Krishnaswamy, 1956).

Nucleotide pyrophosphatase. The reaction was performed as described by Ravindranath & Appaji Rao (1968). The reaction mixture (1 ml) contained sodium barbitone/HCl buffer, pH 9.2 (40 μmol), FAD (0.5 μmol) and enzyme. The reaction mixture was incubated at 37°C for 60 min and the reaction was stopped by the addition of 2 vol. of ethanol. The increase in fluorescence of the reaction mixture (due to the hydrolysis of FAD to FMN) was measured after making up the volume to 5 ml with potassium phosphate buffer, pH 7.0.

FMN hydrolase. The reaction mixture (1 ml) contained FMN (0.1 μmol), Tris/HCl buffer, pH 8.5 (200 μmol), and enzyme. The incubation was performed for 60 min at 37°C and was stopped by the addition of 0.1 ml of 100% trichloroacetic acid. The reaction mixture was transferred to a 98°C water bath for 5 min, and the FMN as well as riboflavin formed were measured after chromatographic separation (Appaji Rao *et al.*, 1963).

Results

Purification

Mung-bean seeds (500 g) were soaked in water for 10 h at 37°C. All subsequent steps were performed at 0–4°C. The seeds were homogenized with 1.5 litres of potassium phosphate buffer, pH 7.4 (0.05 M), in a pre-cooled Waring Blendor for 2 min at 30 s intervals, and the brei was left for 1 h for complete extraction of the enzyme. The homogenate was filtered through cheesecloth ('crude extract', vol. 1775 ml; Table 1) and made up to 10 mM-$MnSO_4$ by the addition of 1.0 M-$MnSO_4$. The suspension was centrifuged at 12000 *g* for 15 min in a Sorvall RC-5B centrifuge. Solid $(NH_4)_2SO_4$ was added to the supernatant solution to achieve a saturation of 30% and stirred for 15 min. The extract was centrifuged for 15 min at 12000 *g* and the supernatant was raised to 60% saturation by a further addition of solid $(NH_4)_2SO_4$ and stirred for 15 min. The precipitate obtained on centrifugation of the extract at 12000 *g* for 15 min was dissolved in buffer A ['$(NH_4)_2SO_4$ fraction', vol. 400 ml; Table 1]. To this solution, a slurry of Blue Sepharose (100 ml of packed volume, approx. 25 g of dry powder) was added and the suspension was stirred for 4–5 h. The Blue Sepharose matrix was collected by centrifugation at 5000 *g* for 2 min and packed into a column (2 cm × 30 cm). The column was washed with 1.0 M-potassium phosphate buffer, pH 7.4, containing 1.0 M-KCl (hereafter referred to as 'buffer B'). The enzyme was eluted with water from the column ('Blue Sepharose eluate', vol. 15 ml; Table 1). The enzyme from the Blue Sepharose column was made up to 0.5 M-potassium phosphate by the addition of buffer A, and was loaded on to a column (1.5 cm × 5.5 cm) of riboflavin–Sepharose pre-equilibrated with buffer A. The column was washed with buffer B. The enzyme was eluted with water. This enzyme ('riboflavin–Sepharose eluate', vol. 5.0 ml; Table 1) was made up to 0.5 M-potassium phosphate and was reloaded on to a column (1.5 cm × 5.5 cm) of C-8 ATP–agarose pre-equilibrated with buffer A. The column was washed with buffer B. The enzyme was eluted with water and the active fractions were pooled ('C-8 ATP–

Table 1. *Purification of mung-bean flavokinase*

Step	Protein (mg)	Activity (units)*	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract	108000	337	0.003	1	100
30–60%-satd.-$(NH_4)_2SO_4$ fraction	13065	209	0.016	5	62
Blue Sepharose eluate	51.3	125	2.430	810	37
Riboflavin–Sepharose eluate	5.0	118	23.600	7867	35
C-8 ATP–agarose eluate	0.31	100	322.500	107500	29

* A unit of activity is defined as 1 nmol of FMN formed/min at 37°C and pH 8.5.

agarose eluate', vol. 1.5ml; Table 1). The enzyme was homogeneous as shown by polyacrylamide-gel electrophoresis at pH 8.6 (Fig. 1 inset). The precedure described above resulted in about 100000-fold purification of the enzyme, with a recovery of approx. 30%. It had no contaminating activity of FAD synthetase, FMN hydrolase, or nucleotide pyrophosphatase, although these activities were detected in the crude extract.

The requirement of orthophosphate for the binding of the enzyme to the affinity matrices is evident from Fig. 1. When the Blue Sepharose eluate was loaded on to a column of riboflavin–Sepharose (Fig. 1), both the enzyme activity (○) and bulk of the protein (□) were obtained in the unadsorbed fractions. When the affinity column was eluted with either 3.0M-KCl. 1mM-riboflavin, 10mM-ATP or water, no enzyme activity was observed in these fractions, although some protein was recovered (results not shown). On the contrary, when the Blue Sepharose eluate was made up to 0.5M-potassium phosphate and then applied to a column of riboflavin–Sepharose pre-equilibrated with buffer A (Fig. 1), a large amount of protein was recovered in the unadsorbed fractions (■); no enzyme activity (●) was detected in them. The protein that was eluted on washing the column with buffer B had no enzyme activity (results not shown). However, when the column was washed with water, immediately after the void volume a protein peak (■) appeared that coincided with the peak of enzyme activity (●). The presence of high concentrations of other salts, such as 3.0M-$(NH)_2SO_4$, -KCl and -NaCl, did not facilitate the binding of the enzyme to the affinity matrices. In view of the higher solubility of potassium phosphate at 0–4°C, this buffer was used in the present study. It was also observed that orthophosphate was necessary for the binding of the enzyme to all the three affinity matrices.

General properties

The enzyme was optimally active in Tris/HCl buffer (pH 8.5–9.0) and at a temperature of 50–55°C. The reaction was linear up to 1h. The molecular weight of the enzyme, measured by using an Ultrogel AcA 34 column (1cm × 60cm) cali-

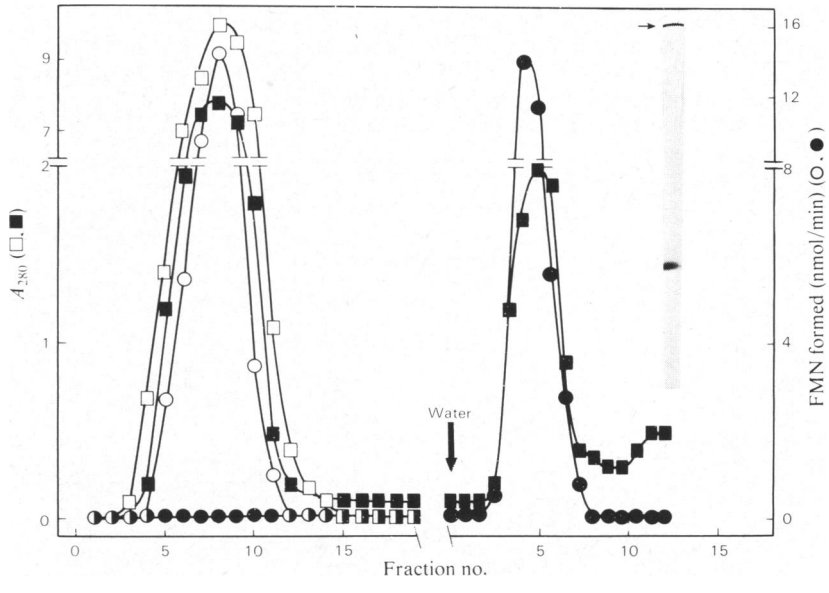

Fig. 1. *Requirement of orthophosphate for the binding of the enzyme to the riboflavin–Sepharose*
Blue Sepharose eluate (34mg, 10ml) was applied to a column of riboflavin–Sepharose equilibrated with water. The column was developed with water. Fractions (2.2ml) were collected after the void volume. The protein (□) and flavokinase activity (○) were measured. In a second experiment, the riboflavin–Sepharose column was equilibrated with buffer A, and the Blue Sepharose eluate (34mg, 20ml; made up to 0.5M-potassium phosphate by adding 10ml of buffer A) was loaded on to it. The column was washed with buffer B. Fractions (2.2ml) were collected, and protein (■) as well as flavokinase activity (●) were measured. The column was eluted with water, and fractions (1.5ml) were collected. Protein (■) and enzymic activity (●) were determined. The inset shows the result of polyacrylamide-gel electrophoresis at pH 8.6.

brated with cytochrome *c* (mol.wt. 14000), chymotrypsin (25000), pepsin (36000), ovalbumin (44000), bovine serum albumin (68000) and hexokinase (102000) as markers, was found to be 35000. The enzyme gave a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

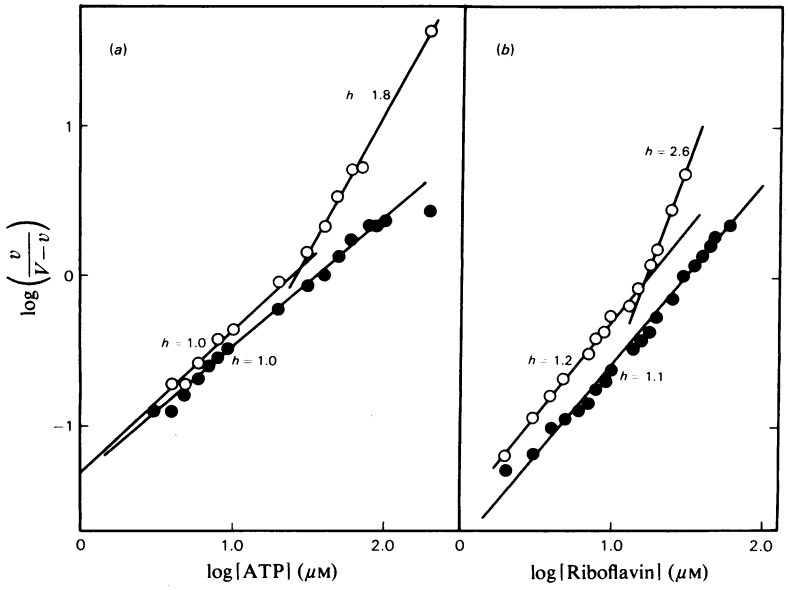

Fig. 2. *Hill plots for the saturation of mung-bean flavokinase with ATP and riboflavin*
(*a*) The riboflavin–Sepharose eluate (25 μg) was incubated with various concentrations of ATP (1–100 μM) at a fixed, but saturating, concentration of riboflavin (100 μM). The FMN formed (○) was measured. The substrate saturation was again determined in the presence of 0.05 M-K_2HPO_4 (●). (*b*) The riboflavin–Sepharose eluate (20 μg) was incubated with various concentrations of riboflavin (1–100 μM) at a fixed, but saturating, concentration of ATP (1 mM). The FMN formed was determined in the absence (○) and presence of 0.1 M-K_2HPO_4 (●).

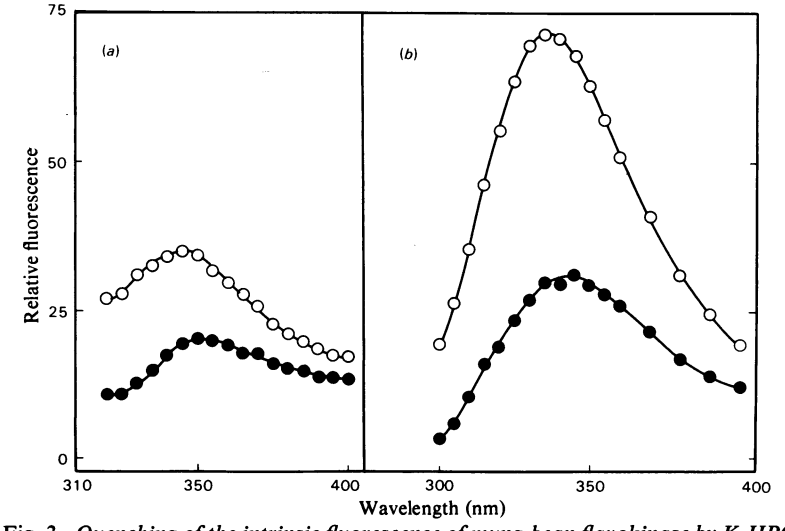

Fig. 3. *Quenching of the intrinsic fluorescence of mung-bean flavokinase by K_2HPO_4*
The C-8 ATP–agarose eluate (30 μg/0.8 ml) was excited at 280 nm (*a*) and 295 nm (*b*) in a Perkin–Elmer model-203 fluorescence spectrophotometer equipped with a 150 W xenon lamp source and an R212 photomultiplier tube. The relative fluorescence (○) was monitored in the range 300–400 nm. The fluorescence spectrum of the enzyme in the presence of 0.1 M-K_2HPO_4 (●) was similarly determined.

corresponding to a mol.wt. of 30000, suggesting that the enzyme may consist of a single polypeptide chain.

Substrate saturation

The break in the Hill plots (Figs. 2*a* and 2*b*) for the saturation of ATP and riboflavin and *h* values of 1 and 2 suggested the prevalance of multiple sites for the interaction of these substrates with the mung-bean flavokinase. In the presence of K_2HPO_4, however, a linear Hill plot with an *h* value of 1 was obtained, suggesting that this ligand interaction with the enzyme caused a conformational change leading to the abolition of co-operative interactions.

Fluorescence

The fluorescence spectrum of the enzyme, excited at 280 and 295 nm, both in the absence and presence of K_2HPO_4 (0.1 M) is shown in Figs. 3(*a*) and 3(*b*). Addition of K_2HPO_4 caused a marked quenching of the emission spectrum at both the excitation wavelengths. There was also a bathochromic shift of about 5 nm of the emission peak when the protein was excited at 280 and 295 nm in the presence of K_2HPO_4 (0.1 M).

Discussion

Flavokinase from rat liver was purified recently by affinity chromatography, and two enzymically active bands were detected on polyacrylamide-gel electrophoresis (Merrill & McCormick, 1980). The presence of a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was the presumptive evidence for the homogeneity of the rat liver enzyme. The plant flavokinase reported here was successfully purified by a three-step affinity procedure. The plant enzyme, unlike that from rat liver, gave a single sharp band on polyacrylamide-gel electrophoresis (Fig. 1, inset). A duplicate gel was sliced into 1 cm pieces and assayed for enzyme activity. The position of the enzymically active slice was identical with the position of the protein band.

The plant enzyme was stable, when stored frozen, up to a few months, unlike the rat liver enzyme, which is unstable, except in the presence of riboflavin and glycerol. The specific activity (nmol of FMN formed/min per mg of protein, 37°C, pH 8.5) of the plant enzyme is 325, compared with the value of 165 for the rat liver enzyme (Merrill & McCormick, 1980). The enzyme had a mol.wt. of 35000 comparable with 28000 for the rat liver enzyme.

The requirement of orthophosphate for the binding of the enzyme to the affinity matrices suggested that the enzyme is a conformationally flexible protein, and the interaction with this ligand leads to the possible formation of a 'dinucleotide fold' or a 'hydrophobic pocket' where ATP, riboflavin or a dinucleotide could bind. Some support for this hypothesis was provided by the observation that the enzyme could also be eluted from Blue Sepharose with β-NAD (1 mM), from riboflavin–Sepharose with riboflavin (1 mM) and from C-8 ATP–agarose with ATP (10 mM). The conformational change occurring on the ligand interactions was evident by the alteration in the environment of tyrosine and tryptophan residues in the enzyme (Figs. 3*a* and 3*b*) and by the alteration in the substrate-saturation pattern (Figs. 2*a* and 2*b*) in the presence of K_2HPO_4. It is interesting to recall the previous observations on the effect of orthophosphate on the activity of nucleotide pyrophosphatase (Krishnan & Appaji Rao, 1972). The interaction of the enzyme with this ion abolished the negative co-operativity exhibited by the enzyme for the binding of FAD and also the heterotropic effects of AMP. The effect of orthophosphate on regulatory enzymes such as AMP nucleosidase (Schramm & Fullin, 1978), adenylate deaminase (Wheeler & Lowenstein, 1979), and its effect on enzyme synthesis when present in the medium (Horiuchi *et al.*, 1959; Torriani, 1960), are well documented.

The present results highlight the conformational flexibility of plant flavokinase and suggest that it could be a regulatory enzyme.

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References

Appaji Rao, N., Cama, H. R., Kumar, S. A. & Vaidyanathan, C. S. (1963) *Biochim. Biophys. Acta* **73**, 87–97

Astrachan, L., Colowick, S. P. & Kaplan, N. O. (1957) *Biochim. Biophys. Acta* **24**, 141–154

Balakrishnan, C. V., Vaidyanathan, C. S. & Appaji Rao, N. (1977) *Eur. J. Biochem.* **78**, 95–102

Bessey, O. A., Lowry, O. H. & Love, R. H. (1949) *J. Biol. Chem.* **180**, 755–769

Burch, H. B., Bessey, O. A. & Lowry, O. H. (1948) *J. Biol. Chem.* **175**, 232–244

Giri, K. V. & Krishnaswamy, P. R. (1956) *J. Indian Inst. Sci.* **38**, 232–244

Giri, K. V., Krishnaswamy, P. R. & Appaji Rao, N. (1957) *Nature (London)* **179**, 1134–1135

Giri, K. V., Krishnaswamy, P. R. & Appaji Rao, N. (1958) *Biochem. J.* **70**, 66–71

Horiuchi, T., Horiuchi, S. & Mizuno, D. (1959) *Nature (London)* **183**, 1529–1530

Kashchenko, V. E. & Shavlovskii, G. M. (1976) *Biokhimiya* **41**, 313–319

Kearney, E. B. & Englard, S. (1951) *J. Biol. Chem.* **193**, 821–834

Krishnan, N. & Appaji Rao, N. (1972) *Arch. Biochem. Biophys.* **149**, 336–348

Kristiansen, T., Sundberg, L. & Porath, J. (1969) *Biochim. Biophys. Acta* **184**, 93–98

Mayhew, S. G. & Wassink, S. H. (1977) *Biochim. Biophys. Acta* **482**, 341–347
McCormick, D. B. (1961) *Proc. Soc. Exp. Biol. Med.* **107**, 784–786
Merrill, A. H., Jr. & McCormick, D. B. (1980) *J. Biol. Chem.* **255**, 1335–1338
Mitsuda, H. (1966) *Proc. Jpn Acad. Sci.* **42**, 940–944
Mitsuda, H., Tomozawa, Y. & Kawai, F. (1963) *J. Vitaminol.* **9**, 142–148
Oya, N. (1968) *Vitamins* **38**, 50–54
Ravindranath, S. D. & Appaji Rao, N. (1968) *Indian J. Biochem.* **5**, 137–142
Ravindranath, S. D. & Appaji Rao, N. (1972) *J. Sci. Ind. Res.* **31**, 210–219
Reddy, A. R. V., Anathanarayanan, V. S. & Appaji Rao, N. (1979) *Arch. Biochem. Biophys.* **198**, 89–96
Reddy, A. R. V., Balakrishnan, C. V., Sobhanaditya, J., Ravindranath, S. D., Ananthanarayanan, V. S. & Appaji Rao, N. (1980) *J. Biosci.* **2**, 211–225
Sadasivan, S. & Shanmughasundaram, E. R. B. (1966) *Enzymologia* **31**, 203–208
Schramm, V. L. & Fullin, F. A. (1978) *J. Biol. Chem.* **253**, 2161–2167
Spencer, R., Fischer, I. & Walsh, C. (1976) *Biochemistry* **15**, 1043–1053
Torriani, A. (1960) *Biochim. Biophys. Acta* **38**, 460–479
Wheeler, T. J. & Lowenstein, J. M. (1979) *J. Biol. Chem.* **254**, 8994–8999