Biochimica et Biophysica Acta, 445 (1976) 622—631

BBA 67933

TRANSAMINASE OF BRANCHED CHAIN AMINO ACIDS

XI. LEUCINE (METHIONINE) TRANSAMINASE OF RAT LIVER MITOCHONDRIA

TATSUHIKO IKEDA, YÔTARO KONISHI and AKIRA ICHIHARA

Institute for Enzyme Research, School of Medicine, Tokushima University, Tokushima 770 (Japan)

(Received April 5th, 1976)

Summary

An aminotransferase (transaminase) which is active for leucine and methionine, but not for valine or isoleucine, was purified from rat liver mitochondria. The purified preparation appeared homogeneous on polyacrylamide disc gel electrophoresis. Its molecular weight was shown to be 55 000 by gel filtration. It differed from enzyme II (leucine aminotransferase, EC 2.6.1.6) in the supernatant fraction, another transaminase which is also specific for leucine and methionine, in molecular weight, K_m values for substrates, electrophoretic mobility, chromatographic behavior and heat stability. From comparison with related transaminases it was concluded to be a new enzyme and named mitochondrial leucine (methionine) transaminase.

Introduction

A series of studies in this laboratory have shown that there are various aminotransferases (transaminases) for branched chain amino acids (enzymes I—III) which are distinguishable chromatographically and/or immunochemically [1]. The supernatant fractions of all rat tissues examined contain enzyme I, and brain, ovary and placenta also contain enzyme III. Enzymes I and III act almost equally well on the three amino acids, valine, leucine and isoleucine, but show very little activity with other amino acids. The best amino acceptor for these enzymes is α-ketoglutarate [2—5], but it is interesting that the keto analogues of the branched chain amino acids are also good amino acceptors [2].

In most tissues these branched chain amino acid transaminases (EC 2.6.1.42) are equally distributed between the supernatant and mitochondrial fractions

[1]. The enzymes I of the supernatant and mitochondrial fractions of hog heart were previously found to have different enzymological properties [6].

Rat liver contains another enzyme (enzyme II, (leucine aminotransferase) EC 2.6.1.6) together with a small amount of enzyme I [4]. This enzyme II in the supernatant fraction was partially purified and shown to be specific for leucine among branched chain amino acids. One third of the leucine specific activity of a homogenate of normal rat liver was found in the supernatant and two thirds in the mitochondrial fraction. However, on administration of a high protein diet or cortisol the specific activity for leucine in the supernatant fraction increased several fold, while that in the mitochondria did not [7].

This paper describes the leucine-specific transaminase from the mitochondrial fraction of rat liver. The enzyme was found to be specific for leucine among branched chain amino acids, but also to act on methionine. Therefore, we reexamined the substrate specificity of enzyme II in the supernatant and found that this enzyme was also active on methionine as well as leucine. However, these enzymes in the supernatant and mitochondrial fractions differed in physical and enzymological properties. Therefore, these two enzymes may be called supernatant and mitochondrial leucine (methionine) transaminase, respectively. However, the exact relationship between them is still unknown, so in this paper the former is referred to as enzyme II and the latter as leucine (methionine) transaminase.

Materials and Methods

Materials

Male Wistar strain rats, weighing 200 to 300 g were given laboratory chow (Oriental Yeast Co., Tokyo) ad libitum before use.

Sephadex G-100 and QAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Uppsala, and hydroxyapatite, DEAE-, and CM-cellulose from Seikagaku Kogyo, Tokyo. Markers for determination of molecular weight were obtained from Mann Research Laboratories, Orangeburg, New York and blue dextran 2 000 from Sigma Chemicals, St. Louis. Norvaline and norleucine were from Nutritional Biochemical Corp., Cleveland, various amino acids from Tanabe Amino Acid Research Foundation, Osaka and DL-ethionine from Wako Pure Chemicals Industries, Osaka. Amicon Ultrafiltration Cells, Model 202 and Dia-Flo Ultrafiltration PM-10 membranes were from Amicon Corp., Lexington and collodion bags from Sartorius-Membran Filter GmbH, Göttingen.

Enzyme assay

Activity of leucine (methionine) transaminase was determined by the method for enzyme I reported before [8], except that the buffer was adjusted pH 8.2. For the usual assay L-leucine and α-ketoglutarate were used as substrates and the α-ketoisocaproate formed was measured as its hydrazone at 440 nm. Enzyme II was assayed as described before [4]. Protein was measured by the method of Lowry et al. [9]. One unit of activity was defined as the amount forming one nmol of keto acid/min and specific activity was expressed as units/mg protein. When indicated, activity was expressed as change of absorbance at 440 nm. In the crude homogenate containing both enzyme I and leucine

(methionine) transaminase the activity of enzyme I was measured as the activity with isoleucine and that of leucine (methionine) transaminase was calculated as the difference between the activities for leucine and isoleucine, as described for assay of enzyme II [4].

Purification of enzyme II

Enzyme II of the supernatant fraction of rat liver was purified as described before [4].

Polyacrylamide disc gel electrophoresis

Electrophoresis was performed by the method of Davis [10] using 7.5% gel and 0.05 M Tris/0.38 M glycine buffer (pH 8.3) and electrophoresis was carried out at 2 mA for 2 h. Proteins were detected by staining the gel with Amidoblack. To locate activity, the disc was cut into 1-mm thick slices and their activities of enzyme II and leucine (methionine) transaminase were measured as described above.

Estimation of the molecular weights of enzyme II and leucine (methionine) transaminase

5 ml of purified enzyme were applied to a Sephadex G-100 column (2.8 × 80 cm) equilibrated with 0.1 M phosphate buffer (pH 7.8) at 4°C. Fractions of 3 ml were collected. Blue dextran 2000 was used as a marker of the void volume. The following markers of known molecular weight were used: cytochrome *c* (M_r = 12 400), myoglobin (M_r = 17 800), chymotrypsinogen A (M_r = 25 000), ovalbumin (M_r = 45 000) and bovine albumin (M_r = 67 000). The molecular weights of enzyme II and leucine (methionine) transaminase were calculated by the method of Andrews [11].

Results and Discussion

Purification of leucine (methionine) transaminase

The mitochondrial fraction was prepared from the livers of 20 rats by the method of Hogeboom [12] and suspended in 10 mM potassium phosphate buffer (pH 7.8) containing 5 mM β-mercaptoethanol, 0.01 mM pyridoxal phosphate and 1 mM EDTA (Step I). All buffers used were prepared from potassium salts and contained these three components. The protein concentration was adjusted to about 20 mg/ml. The suspension was freezed-thawed 3 or 4 times, and the resulting suspension of fragmented mitochondria was centrifuged at 105 000 × *g* for 20 min; the supernatant (Step II) was fractionated by adding solid ammonium sulfate; the material precipitated with between 35 and 85% saturation of salt was dissolved in a small amount of 5 mM phosphate buffer (pH 7.8) and dialyzed against the same buffer overnight. The dialyzed preparation was applied to a DEAE-cellulose column (3.6 × 40 cm) previously equilibrated with the same buffer. For all subsequent column chromatographies the columns used were previously equilibrated with the same buffer as that in which the enzyme was dissolved. The unadsorbed fraction obtained by washing the column with 700 ml of the same buffer was collected. Enzyme I was adsorbed on DEAE-cellulose; leucine (methionine) transferase was not (Fig. 1).

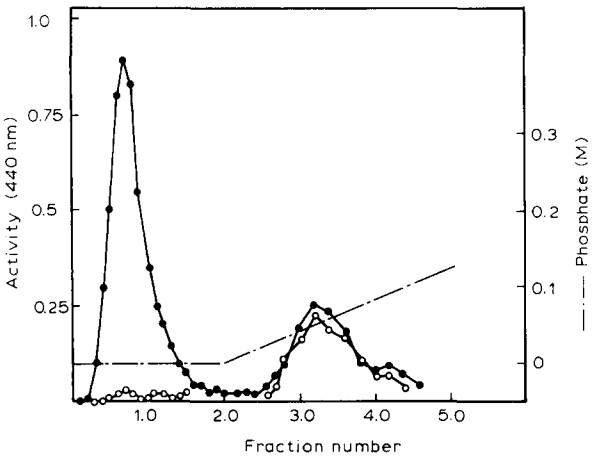

Fig. 1. DEAE-cellulose column chromatography of transaminase activities for leucine from rat liver mitochondria. Activity appeared in the unadsorbed fractions with 5 mM phosphate buffer (pH 7.8) and in fractions eluted with 20 mM phosphate buffer in a linear gradient of buffer. ●———●, activity for leucine; ○———○, activity for isoleucine.

Unadsorbed material was fractionated by addition of solid $(NH_4)_2SO_4$. Material precipitated with 40—80% saturation of salt was dissolved in a small amount of 20 mM phosphate buffer (pH 7.9) containing the three additions described above and 10% glycerol and dialyzed against the same buffer overnight. The dialyzed fraction (Step III) was applied to a hydroxyapatite column (2 × 20 cm). The column was eluted with 400 ml of a linear gradient of 20 mM to 0.2 M phosphate buffer (pH 7.0). Alanine transaminase (EC 2.6.1.2) was separated from the enzyme by this procedure. Fractions with transaminase activity were combined (step IV) and concentrated in an Amicon Ultrafiltration Cell, Model 202 using a PM-10 membrane. The concentrated solution was applied to a Sephadex G-100 column (2.8 × 75 cm) equilibrated with 0.1 M phosphate buffer (pH 7.8) and the column was eluted with 300 ml of the same buffer. Fractions with activity were mixed with solid ammonium sulfate. Material precipitating with between 55 and 65% saturation of salt was dissolved in 10 mM Tris · HCl buffer (pH 6.8) containing 10% glycerol and dialyzed against the same buffer for 8 h. The dialyzed solution (Step V) was applied to a CM-cellulose column (1.8 × 10 cm) and the column was eluted with 500 ml of a linear gradient of 0 to 0.15 M KCl. Fractions with activity were combined, concentrated in a collodion bag in a Sartorius-Membran Filter, and dialyzed against 50 mM pyrophosphate buffer (pH 8.2) containing 10% glycerol. The dialyzed preparation (Step VI) was applied to a QAE-Sephadex A-50 column (1.8 × 14 cm) and the unadsorbed fraction was concentrated in an Amicon Ultrafiltration cell, Model 202 (Step VII). This preparation was used for subsequent experiments unless otherwise indicated. Results at each step of purification are shown in Table I. The enzyme was purified about 200-fold from the mitochondrial suspension and appeared nearly homogeneous on polyacrylamide disc gel electrophoresis (Fig. 2). The enzyme activity coincided with this main band of protein. The yield of activity by this purification procedure was rather small, owing to the low stability of the enzyme after the stage of DEAE-cellulose col-

TABLE I

PURIFCATION OF LEUCINE (METHIONINE) TRANSAMINASE FROM RAT LIVER MITOCHONDRIA

Step	Procedure	Total volume (ml)	Protein (mg/ml)	Specific activity (units/mg protein)	Yield (%)	Met/Leu activity ratio
I	Mitochondrial suspension	730	18.7	0.7	100	1.3
II	Mitochondrial extract	680	9.6	1.6	119	1.0
III	DEAE-cellulose and ammonium sulfate	148	14.3	2.7	64	1.1
IV	Hydroxy-apatite	36	5.1	10.3	21	0.9
V	Sephadex G-100 and ammonium sulfate	32	1.2	29.5	13	0.9
VI	CM-cellulose	12	0.6	77.1	6	1.1
VII	QAE-Sephadex A-50	5	0.4	137.3	3	

umn chromatography, particularly in the frozen state (Table II). It was found that addition of 10% glycerol and pyridoxal phosphate stabilized the enzyme as shown in Table II. It should be mentioned that the maximum specific activity achieved for leucine (methionine) transaminase was one tenth of that obtained for enzyme II [4].

Comparison of the properties of the mitochondrial leucine (methionine) transaminase and enzyme II from the supernatant

Sephadex G-100 filtration showed that the molecular weight of the leucine (methionine) transaminase was 55 000, whereas that of enzyme II was 65 000. The K_m values of leucine (methionine) transaminase for substrates were calculated from Lineweaver-Burk plots as 1.49 mM for leucine and 0.2 mM for α-ketoglutarate. Those of enzyme II were previously found to be 25 mM and 0.07 mM, respectively [4]. It is interesting that the K_m values of other transaminases, which are distributed in both the supernatant and mitochondrial fractions of tissues, for substrates such as aspartate, alanine and branched chain amino acids (enzyme I), show similar tendencies to those found in this paper, i.e. the K_m values for amino donors of supernatant enzymes are significantly higher than those of the mitochondrial enzymes, while the K_m values of the enzymes in the two fractions for amino acceptors show the reverse relation (see discussion in ref. 6). The heat stabilities of the enzymes in the two fractions were also different: enzyme II was quite stable on heating at 45°C for 1 h, while the activity of leucine (methionine) transaminase decreased linearly with time and half the activity was lost in 40 min. These findings are similar to those in hog heart in which the supernatant isozymes of aspartate aminotransferase

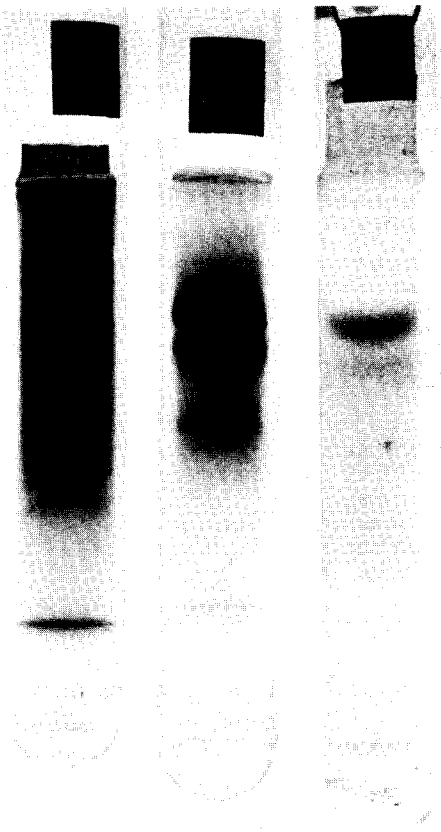

Fig. 2. Polyacrylamide disc gel electrophoresis of leucine (methionine) transaminase. 1, Step III preparation (100 μg of protein); 2, Step VI preparation (75 μg of protein); 3, Step VII preparation (50 μg of protein).

(EC 2.6.1.1) and branched chain amino acid aminotransferase (enzyme I) were found to be more stable than those of the mitochondrial isozymes (see discussion in ref. 6). These common differences in the properties of isozymes in the two fractions suggest that mitochondrial enzymes may become unstable when they are solubilized, because their conformations change, and this may also induce a change of K_m values. Alternatively, isozymes in the supernatant and mitochondrial fractions, respectively, may have certain specific structures which suit them to their locations.

There are other differences in the properties of enzyme II and leucine (methionine) transaminase. The optimal pH of leucine (methionine) transaminase was 8.2, whereas that of enzyme II was 8.7 as reported before [4]. The electrophoretic mobilities of the two enzymes on disc gel electrophoresis were also different, as shown in Fig. 3.

The apoenzyme was prepared by dialysis of the holoenzyme against buffer containing 5 mM hydroxylamine as reported before [8]. In this way the activity was greatly reduced, but it could be partially, though not completely recovered by addition of pyridoxal phosphate (data not shown). This low recov-

TABLE II

STABILITY OF LEUCINE (METHIONINE) TRANSAMINASE

Enzyme (Step IV) was stored at 4°C for 4 days or −20°C for 24 h with the indicated supplements. Activities were expressed as percentages of the original activity.

Storage	Supplements	Activity (%)
4°C for 4 days	5 mM phosphate buffer (pH 7.8)	58.1
	100 mM phosphate buffer (pH 6.2)	33.7
	100 mM phosphate buffer (pH 7.8)	65.0
	100 mM phosphate buffer (pH 7.8) + 0.1 mM pyridoxal phosphate	88.1
	100 mM phosphate buffer (pH 7.8) + 0.1 mM pyridoxal phosphate + 10% glycerol	95.0
−20°C for 24 h	5 mM phosphate buffer (pH 7.8)	4.8
	100 mM phosphate buffer (pH 7.8)	10.0
	100 mM phosphate buffer (pH 7.8) + 0.1 mM pyridoxal phosphate	38.1
	100 mM phosphate buffer (pH 7.8) + 0.1 mM pyridoxal phosphate + 10% glycerol	92.5

ery may have been because the apoenzyme is unstable and is inactivated during dialysis. It has been shown that β-mercaptoethanol activates enzyme I [2,3,6], but not enzyme II [4]. Leucine (methionine) transaminase was activated about 20% by 10 mM β-mercaptoethanol or dithiothreitol (data not shown).

Comparison of the substrate specificities of leucine (methionine) transaminase and related transaminases

The substrate specificity of the mitochondrial enzyme for amino donors is

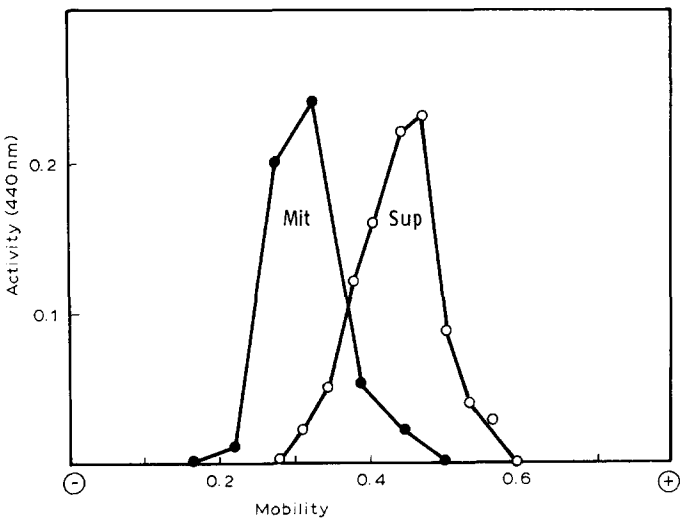

Fig. 3. Electrophoretic mobilities of leucine (methionine) transaminase and enzyme II. The procedure is described in Materials and Methods. ●———●, leucine (methionine) transaminase; ○———○, enzyme II.

shown in Table III. It is interesting that the enzyme in the mitochondrial fraction was specific for leucine among branched chain amino acids, but that it showed highest activity for ethionine and methionine. The ratio of its activities for leucine and methionine during purification was quite constant, as shown in Table I. It also showed some activity with norvaline, norleucine and homocysteine, but not with arginine, lysine, ornithine or homoserine. The ratio of the activities of the supernatant enzyme II for leucine and methionine was 1.2 at the various steps of purification reported before [4] (data not shown). Thus enzyme II seems to have the same substrate specificity as leucine (methionine) transaminase, although it differs from the latter in many properties as described above. However, it is still uncertain whether the different properties of these two enzymes are due to differences in their protein moieties or of other components, but further chemical and immunological studies are needed on this.

It is also interesting that the specificity of the activator of prephenate dehydratase (EC 4.2.1.51) of *Bacillus subtilis* is very similar to that of leucine (methionine) transaminase [13]. These amino acids all have a hydrophobic region, including a sulfur atom, and hence this region must be important for enzyme activity.

The possibility that basic amino acids, such as arginine, ornithine and lysine, were actually transaminated, but that the hydrazones of their keto acids could not be extracted by the present assay method, was examined by testing the possible competitive effects of basic amino acids and leucine for the active site (Table IV). The results showed that these amino acids did not reduce the activity with leucine, indicating that they are not transaminated. Moreover, ornithine—oxo-acid aminotransferase (EC 2.6.1.13) is inhibited by branched chain amino acids and these do not serve as substrates [14,15]. Branched chain amino acid transaminase (enzymes I and III) is active with all three branched chain amino acids as well as with norleucine and norvaline [2,3, 6,8], and shows slight activity with methionine, but it does not act on

TABLE III

SUBSTRATE SPECIFICITY OF LEUCINE (METHIONINE) TRANSAMINASE

Concentrations of 20 mM L-amino acids and 40 mM DL-amino acid were used. All amino acids were L-form unless otherwise specified. Activities are expressed as percentages of that with L-leucine.

Amino acids	Relative activity (%)
DL-Ethionine	198
Methionine	103
Leucine	100
Norvaline	87
Norleucine	50
Homocysteine	42
Valine, isoleucine, DL-α-Aminobutyrate, threonine, homoserine, cysteine, serine, glycine, tyrosine, tryptophan, phenylalanine, lysine, arginine and ornithine	<10

TABLE IV

EFFECTS OF BASIC AMINO ACIDS ON ACTIVITY OF LEUCINE (METHIONINE) TRANSAMINASE

The reaction mixture was as described in Materials and Methods, except that 20 mM basic amino acids were preincubated with the enzyme for 5 min before addition of 5 mM leucine. The reaction was carried out for 20 min. Activities are expressed as in Table III.

Addition	Activity (%)
None	100
L-Arginine	95
L-Lysine	90
L-Ornithine	90

homocysteine at all [2]. The mitochondrial fraction shows quite high activities of aspartate and alanine transaminases, but alanine transaminase was clearly separated from leucine (methionine) transaminase by hydroxyapatite column chromatography. Aspartate transaminase in mitochondria also shows activity with aromatic acids, but not leucine [16]. Moreover, previous work showed that purified aspartate and alanine transaminases from hog heart showed no activity for leucine [8]. Glutamine—oxo-acid aminotransferase (EC 2.6.1.15) is also found in both the mitochondrial and supernatant fractions [17] and shows activity with methionine and ethionine, but not leucine [18]. It also shows some activity with cysteine, whereas leucine (methionine) transaminase does not. Moreover, it is adsorbed on a DEAE-cellulose column under similar conditions to those used in this work [17] and is heat stable [18] and its molecular weight was reported to be 110 000 [18]. Thus it is clearly different from leucine (methionine) transaminase.

Bacterial transaminases with similar substrate specificities to leucine (methionine) transaminase have been reported. For instance, in *Escherichia coli*, transaminase A shows activity with leucine and methionine, but it is much more active with aromatic amino acids [19]. Leucine-pyruvate transaminase of *Gluconobacter suboxydans* shows highest activity with methionine and leucine, but it also has some activity for aromatic amino acids [20]. A mitochondrial transaminase of *Neurospora crassa* shows highest activity with leucine and lower activities with valine and isoleucine, but it has no activity with methionine [21]. From these findings on related transaminases in mammals and microorganisms it is concluded that leucine (methionine) transaminase is a new transaminase.

The physiological significance of this enzyme is not clear. It is not inducible by dietary or hormonal treatment of animals [7], although administration of protein-free diet for a long period is reported to decrease its activity [22]. Leucine is exclusively oxidized in mitochondria [23] and hence it is conceivable that the transaminase for leucine in the cytosol functions in adjusting the amino acid concentration for protein synthesis, whereas that in the mitochondria functions in metabolism of the amino acid. The metabolism of leucine in the liver may be low [24], but the concentration of this amino acid in the liver may be very important for urea synthesis as well as for protein synthesis: the concentration of leucine regulates the activities of both glutamate dehydrogenase (EC 1.4.1.3) and ornithine—oxo-acid aminotransferase [14,15,25,26].

It is thought that methionine is mainly metabolized by the cystathionine pathway, so the physiological significance of a transaminase of methionine is uncertain. Methylmercaptan and α-ketobutyrate are formed by the pathway involving transamination and it has been reported that methylmercaptan is produced in severe liver diseases [27]. We have not examined the reversibility of the reaction catalyzed by leucine (methionine) transaminase, but it was shown that that of enzyme I is fully reversible [28] and nutritional studies have shown that keto analogues of leucine or methionine are equivalent to these amino acids for animal growth [29] suggesting that the reaction of this enzyme may be partially if not completely, reversible.

Acknowledgements

We are grateful to Drs. Chiseko Noda and Katsuyuki Imai for their helpful advice. This work was supported in part by a grant for Scientific Research from the Ministry of Education, Science and Culture.

References

1 Ichihara, A. (1975) Ann. N.Y. Acad. Sci. 259, 347—354
2 Taylor, R.T. and Jenkins, W.T. (1966) J. Biol. Chem. 241, 4396—4405
3 Aki, K., Yokojima, A. and Ichihara, A. (1969) J. Biochem. 65, 539—544
4 Aki, K., Ogawa, K. and Ichihara, A. (1968) Biochim. Biophys. Acta 159, 276—284
5 Krebs, H.A. (1975) Advan. Enzyme Regul. 13, 449—472
6 Aki, K., Ogawa, K., Shirai, A. and Ichihara, A. (1967) J. Biochem. 62, 610—617
7 Ichihara, A., Takahashi, H., Aki, K. and Shirai, A. (1967) Biochem. Biophys. Res. Commun. 26, 674—678
8 Ichihara, A. and Koyama, E. (1966) J. Biochem. 59, 160—169
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
10 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
11 Andrews, P. (1964) Biochem. J. 91, 222—233
12 Hogeboom, G.H. (1955) Methods Enzymol. 1, 16—19
13 Pierson, D.L. and Jensen, R.A. (1974) J. Mol. Biol. 90, 563—579
14 Katunuma, N., Matsuda, Y. and Tomino, I. (1964) J. Biochem. 56, 499—503
15 Strecker, H.J. (1965) J. Biol. Chem. 240, 1225—1230
16 Miller, J.E. and Litwack, G. (1971) J. Biol. Chem. 246, 3234—3240
17 Yoshida, T., Towatari, T. and Katunuma, N. (1968) in International Symposium on Pyridoxal Enzymes (Yamada, K., Katunuma, N. and Wada, H., eds.), pp. 63—66, Maruzen, Co., Tokyo
18 Cooper, A.J.L. and Meister, A. (1972) Biochemistry 11, 661—670
19 Rudman, D. and Meister, A. (1953) J. Biol. Chem. 200, 591—607
20 Tachiki, T. and Tochikura, T. (1975) Biochim. Biophys. Acta 397, 342—346
21 Collins, M. and Wagner, R.P. (1973) Arch. Biochem. Biophys. 155, 184—193
22 McFarlane, I.G. and von Holt, C. (1969) Biochem. J. 111, 565—571
23 Noda, C. and Ichihara, A. (1974) J. Biochem. 76, 1123—1130
24 Noda, C. and Ichihara, A. (1976) J. Biochem., in the press
25 McGivan, J.D., Bradford, N.M., Crompton, M. and Chappell, J.B. (1973) Biochem. J. 134, 209—215
26 Mendes-Mouras, J., McGivan, J.D. and Chappell, J.B. (1975) Biochem. J. 146, 457—464
27 Meister, A. (1965) in Biochemistry of Amino Acids, Vol. II, pp. 785—786, Academic Press, New York
28 Ogawa, K. (1971) Japan. J. Biochem. Soc. (in Japanese) 43, 197—209
29 Meister, A. (1965) in Biochemistry of Amino Acids, Vol. I, pp. 220—224, Academic Press, New York