Biochimica et Biophysica Acta, 614 (1980) 294—302

BBA 69058

PROLINE OXIDASE INHIBITION BY FREE FATTY ACIDS OF RAT PANCREAS

HIROKO KADOWAKI, GEORGE M. PATTON [a] and W. EUGENE KNOX *

Department of Biological Chemistry, Harvard Medical School and the Cancer Research Institute of the New England Deaconess Hospital, Boston, MA 02215 and [a] Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02154 (U.S.A.)

(Received December 11th, 1979)

Key words: Proline oxidase inhibition; Free fatty acid; (Rat pancreas)

Summary

Proline oxidase activity was not measurable in pancreas homogenate but was measurable in pancreas slices. Moreover, added pancreas homogenate inhibited proline oxidase activity in rat liver mitochondria and several other tissues. The partially purified inhibitor from pancreas also inhibited the activity of glutamate, glucose 6-phosphate, NADH and succinate dehydrogenases. The inhibition was reversed by the addition of bovine serum albumin. Further studies to identify the inhibitor indicated that it consisted of free fatty acids that were enzymatically released after homogenization of pancreas. The free fatty acids released appeared to be derived primarily from triglycerides.

Proline oxidase activity in liver, kidney, heart and brain increases after birth and reaches its highest level in adult rat liver. In pancreas and small intestine, on the other hand, the activity of the enzyme decreases after birth and is not detectable in tissue homogenates later than 2 weeks postpartum [1,2]. Moreover, pancreas and small intestine homogenates from adult rats inhibit proline oxidase activity in liver preparations. The possibility that this inhibition exerts a regulatory effect on proline metabolism prompted us to investigate the inhibition of liver proline oxidase by pancreas homogenate.

* To whom correspondence should be addressed.
The research described in the paper involved animals maintained in animal care facilities fully accredited by the American Association for Accrediation of Laboratory Animal Care. This is Article 664 from the Cancer Research Institute of the New England Deaconess Hospital.

Materials and Methods

Materials

Animals. Male, inbred Kx (formerly NEDH) rats were fed Purina rat chow and water ad libitum and killed between 90—100 days of age by cervical dislocation.

Chemicals. DL-Δ^1-Pyrroline-5-carboxylic acid was prepared by the method of Mezl and Knox [3]. Baker's yeast glucose-6-phosphate dehydrogenase, NADH, NAD, cytochrome *c*, trypsin, *p*-tosyl-L-arginine methyl ester, palmitoyl-CoA and free fatty acids were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A.

Methods

All procedures were performed at 0—4° C unless stated otherwise.

Rat liver fractionation. The livers were homogenized in 9 vols. (w/v) of 0.25 M sucrose containing 0.05 M potassium phosphate buffer (pH 7.5). The twice-washed mitochondrial fraction was suspended in 0.05 M potassium phosphate buffer (pH 7.50) to make a final protein concentration of 5 mg/ml, sonicated for 2 min with a Fisher Ultrasonic Probe, Model BP-2, and centrifuged at 105 000 × *g* for 60 min for the preparation of the liver mitochondrial membrane and matrix fractions.

Unwashed mitochondria were stored as the source of proline oxidase, and other fractions were used for testing the inhibitor on a variety of enzyme activities. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was measured in the cytosol fraction [4,5]. Δ^1-Pyrroline-5-carboxylate reductase (EC 1.5.1.2) [6] was measured in the cytosol fraction which was prepared at 25° C. Ornithine aminotransferase (EC 2.6.1.13) [7] and cytochrome *c* oxidase (EC 1.9.3.1) [8] were measured in washed mitochondria. The mitochondrial matrix fraction was used for measurements of glutamate dehydrogenase (EC 1.4.1.2) in both directions [9,10], Δ^1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12) [1], and malate dehydrogenase (EC 1.1.1.37) [11]. The mitochondrial membrane fraction was used for measurements of succinate dehydrogenase (EC 1.3.99.1) [12] and NADH dehydrogenase (EC 1.6.99.3) [13].

Protein was measured with bovine serum albumin as standard [14].

Proline oxidase and its inhibitor. The unwashed liver mitochondrial fraction was resuspended in 0.05 M potassium phosphate buffer (pH 7.5). Its proline oxidase activity was stable when stored at −20° C. Portions containing 1.5 mg protein (with activity of 0.8 μmol of product formed in the assay) were assayed aerobically in phosphate buffer at pH 8.0 with added cytochrome *c* at 37° C for 30 min [1]. The product, pyrroline 5-carboxylate, was measured with *o*-aminobenzaldehyde and the activity expressed in units of μmol/min. At least three amounts of an inhibitor preparation that gave between 75 and 25% of the control activity were added 10 min before the reaction was started by proline addition. The amount that by interpolation gave 50% inhibition was designated 1 unit of inhibitor. Amounts of inhibitor are expressed as units/mg pancreas equivalent weight.

Extraction and separation of lipid fractions. Lipids were extracted from tissues and washed by the method of Folch et al. [15]. The chloroform was

evaporated under N_2 gas and the extract redissolved in light ether (petroleum ether), b.p. 38—57°C (one half the chloroform/methanol volume). Undissolved material in the light ether was removed by centrifugation. The light ether fraction was washed twice with 0.5 vols. 0.01 N HCl, and was then extracted three times with 0.3—0.4 vols. 0.2 M Na_2CO_3. The Na_2CO_3 fraction was adjusted to pH 2.0 with HCl and extracted three times with 5 ml light ether which was evaporated under N_2. For analyses the methyl esters were prepared as described by Wadke et al. [16].

Quantitative analysis of fatty acid methyl esters was performed by gas chromatography as described by Patton and Lowenstein [17]. Relative response factors were determined with NU-CHEK-PREP (Elysian, MN, U.S.A.) quantitative standards 2A and 3A′. Pentadecanoic acid ($C_{15:0}$) was used as the internal standard.

Results

Assay and properties of inhibitor from pancreas

Inhibition of rat liver proline oxidase was observed only with homogenates of pancreas (1 unit of inhibitor in 0.3—0.5 mg tissue) and of small intestine (1 unit in 5 mg tissue). Homogenates of other adult rat tissues (spleen, skeletal muscle, submaxillary gland, lung), 19-day fetal tissues (brain, heart, lung) and transplanted rat tumors (of mammary gland [18] and liver [19]) were like pancreas in having no measurable proline oxidase activity, but as much as 10 mg of each did not inhibit liver proline oxidase activity. On the other hand, homogenates of several tissues with proline oxidase activity (liver, kidney, heart, brain) were all inhibited equally by the added inhibitor from pancreas.

The stable particulate preparation of liver proline oxidase provided a sensitive and convenient assay of the inhibitor from pancreas. Its enzyme activity was more sensitive to pancreas inhibition than the homogenate preparation, since the inhibition was inversely proportional to the total protein present in the assay. Bovine serum albumin added to washed liver mitochondria largely prevented the inhibition and also reversed it equally well after the inhibition

TABLE I

EFFECT OF ALBUMIN ON PROLINE OXIDASE INHIBITION

Various amounts of the partially purified pancreas inhibitor and a fixed amount of liver mitochondrial fraction (1.5 mg protein) were preincubated at 0°C for 10 min before the addition of substrate to begin the reaction at 37°C for 30 min. For prevention of the inhibition, 10 mg bovine serum albumin were added before the inhibitor. For reactivation, the substrate and albumin were added together after the preincubation. The amount of albumin which was required for maximum reactivation was estimated graphically.

Pancreas equivalent in assay (mg)	Proline oxidase activity (μmol/assay)		
	Inhibition (No albumin added)	Prevention (Albumin (10 mg) preincubated)	Maximum reactivation (Albumin (mg) added after preincubation)
0	0.700	0.728	0.700 with 0 mg
1	0.259	0.619	0.544 with 2 mg
2	0.077	0.512	0.448 with 4 mg
4	0.009	0.441	0.324 with 8 mg
6	0.000	0.387	0.207 with 10 mg

was fully developed (Table I). About 2 mg albumin were required to oppose the inhibitor from 1 mg pancreas.

Detection of proline oxidase activity in adult pancreas was possible for the first time using conditions that minimized the action of the inhibitor. Pancreas slices (approx. 0.5 mm thickness) exhibited proline oxidase activity in the presence or absence of albumin, as did homogenates when prepared in albumin. The activity was about 0.1 unit/g in pancreas compared to 4.5 units/g in rat liver homogenates. Still lower proline oxidase activities were found in slices of adult small intestine.

Some other soluble and insoluble enzymes (Table II) were about equally as sensitive as proline oxidase to inhibition by the partially purified inhibitor from pancreas. The sensitivity to inhibition was greatest in those enzyme assays containing the least protein, such as those of yeast glucose-6-phosphate or rat liver succinate dehydrogenases. The degree of inhibition of glucose-6-phosphate dehydrogenase could be lessened by higher concentrations of cofactors as well as protein in the same way as observed by Taketa and Pogell for inhibition by palmitoyl-CoA [4]. Cofactor effects presumably also accounted for the fact that glutamate dehydrogenase acting in the direction of oxidation was 2.5-times more sensitive to inhibition than the reductive reaction with the same amounts of protein in each assay. The inhibitions of both glutamate and glucose-6-phosphate dehydrogenases were completely prevented by added albumin. Other soluble and insoluble enzymes, pyrroline-5-carboxylate reductase, ornithine aminotransferase, pyrroline-5-carboxylate dehydrogenase, cytochrome *c* oxidase and malate dehydrogenase, were not inhibited with as much as 6—12 mg equivalents of pancreas.

The nonspecific inhibition of a number of enzymes, to some degree conditioned by protein and cofactor concentrations and prevented by albumin, is also produced by low concentrations of palmitoyl-CoA [4]. Palmitoyl-CoA also inhibited liver proline oxidase [20] and in our assay the inhibition by 55 μg

TABLE II

ENZYMES INHIBITED BY PARTIALLY PURIFIED PANCREAS INHIBITOR

Enzyme	Protein in assay (μg)	Activity * in assay (nmol/min)	Pancreas equivalent for 50% inhibition (mg)
Glutamate dehydrogenase			
Glutamate oxidation	140	8	0.45
α-Ketoglutarate reduction	140	78	1.10
Glucose-6-phosphate dehydrogenase			
Yeast **	0.14	10	0.20
Liver cytosol **	210	3	2.00
Liver cytosol ***	1170	9	27.36
Succinate dehydrogenase	7	0.25	0.62
NADH dehydrogenase	70	39	2.84

* Without addition of pancreas inhibitor.

** Assay condition [11]: 0.05 M Tris-HCl buffer (pH 7.5)/NADP, 0.15 mM/glucose 6-phosphate, 0.1 mM.

*** Assay condition [12]: 0.05 M triethanolamine buffer (pH 7.6)/NADP, 0.5 mM/glucose 6-phosphate 3.3 mM.

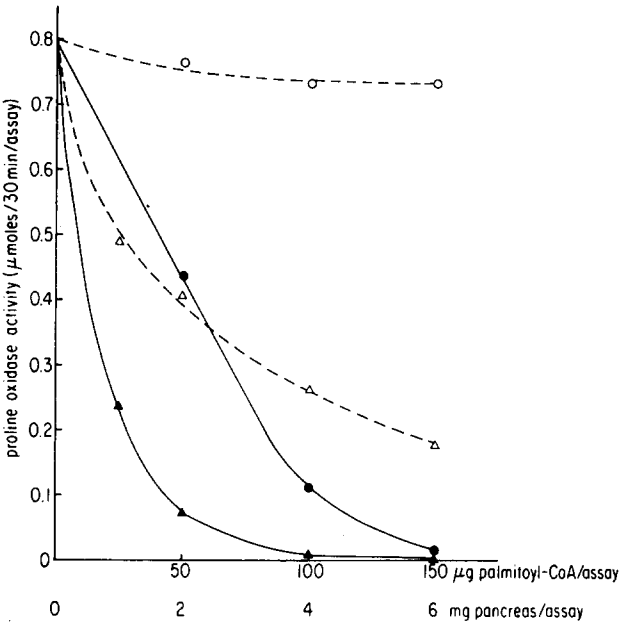

Fig. 1. The effect of alkaline cysteine treatment of partially purified pancreas inhibitor and palmitoyl-CoA on the inhibition of proline oxidase activity. Partially purified pancreas inhibitor and palmitoyl-CoA were incubated at 38°C for 60 min with 0.02 M cysteine in 0.1 M glycine buffer (pH 9.0). After incubation, the pH was readjusted to 8.0 for the assay of proline oxidase. Partially purified pancreas inhibitor, ▲——▲; alkaline cysteine-treated partially purified pancreas inhibitor, △- - - - -△; palmitoyl-CoA, ●——●; and alkaline cysteine-treated palmitoyl-CoA, ○- - - - -○.

palmitoyl-CoA was equivalent to that of 1 pancreas unit. However, decomposition of palmitoyl-CoA by alkaline treatment with cysteine [21] destroyed its inhibition of proline oxidase but the same treatment only partially affected the inhibition by the pancreas extract (Fig. 1).

Identification of inhibitor in lipid fractions

The inhibitor was present almost wholly in the several particulate fractions [22] of pancreas homogenate, and not preferentially in any, including the zymogen granules marked by trypsin assays [23—24]. A partially purified and temporarily soluble inhibitor fraction was prepared in 50% yield by sonication at pH 9.0 of the entire particulate fraction of pancreas followed by centrifuging off the insoluble debris. This preparation was stable to treatment at 100° C for 10 min and to prolonged storage at 0° C, although it formed a precipitate at pH 7.0 that exhibited anisotropy. The inhibitor was not diffusible when dialyzed.

These properties of the inhibitor and the similarity of its action to that of palmitoyl-CoA led to the finding that substantially all of the partially purified inhibitor as well as that in crude pancreas homogenate could be extracted into chloroform/methanol, and most of it then partitioned into the free fatty acid fraction with an overall recovery of 40% (Table III). The major loss occurred at the Folch wash of the chloroform/methanol fraction with 0.9% KCl. None of the lost inhibitor was recovered from this aqueous wash or from the recombined fractions.

TABLE III

INHIBITION OF PROLINE OXIDASE BY LIPID FRACTIONS FROM PANCREAS HOMOGENATE

Organic solvents were evaporated under N_2 gas and the residue was suspended in water for the assay.

Fraction	Inhibitor (units/mg pancreas equivalent)	Recovery (%)
Homogenate	2.00	100.0
Chloroform/methanol extract	1.67	83.5
Chloroform/methanol insoluble	0.12	6.0
Folch wash (0.9% KCl/methanol)	0.00	0.0
Chloroform layer	0.95	47.5
Dried chloroform layer:		
Light ether soluble	0.91	45.5
Light ether insoluble	0.14	7.0
Aqueous phase (0.2 M Na_2CO_3):		
Light ether phase	0.17	8.5
Light ether extract from acidified aqueous phase	0.81	40.5

Isolation of the inhibitor directly from intact pancreas by solvent extraction revealed that a short incubation of the disintegrated tissue in an aqueous medium was necessary to form the inhibitor. Even in aqueous homogenates the yield was increased with time of standing beyond 5 min at 37° C. The process of formation was heat labile and presumably enzymic, since the inhibitor in incubated homogenate survived heating but was not produced from pancreas that had been heated before it was homogenized (Table IV).

Incubation of pancreas homogenate produced parallel increases of more than 15-fold in the amounts of inhibitor and of total free fatty acids. The amount of fatty acids present per unit of inhibition decreased slightly with incubation, and the relative proportion of unsaturated fatty acids increased (Table V), which indicated that unsaturated fatty acids were somewhat more inhibitory than saturated ones. Analysis of the inhibitions produced by each of the pure fatty acids showed that this was so and that stearic acid was a uniquely poor

TABLE IV

FORMATION OF INHIBITOR IN INCUBATED PANCREAS HOMOGENATE

Pancreas was homogenized directly in 30 vols. of chloroform/methanol (2:1), or was homogenized in 9 vols. of aqueous sucrose/phosphate buffer at 0—2°C, and incubated at 37°C for various times before chloroform/methanol extraction.

Homogenizing medium	Incubation at 37°C (min)	Inhibitor extracted (units/mg pancreas equivalent)
$CHCl_3$/MeOH	None	0.04
Aqueous	0	0.40
Aqueous	5	1.25
Aqueous	30	1.43
Aqueous	60	2.00
Aqueous	60, then 10 min at 100°C	2.22
Aqueous, of heated pancreas *	60	0.02

* Intact pancreas was heated at 100°C for 10 min followed by homogenization and incubation at 37°C.

TABLE V

EFFECT OF INCUBATION ON FREE FATTY ACIDS AND INHIBITOR ISOLATED FROM PANCREAS BY SOLVENT EXTRACTION

Intact pancreas was disintegrated in 30 vols. of chloroform/methanol (2:1) immediately after excision. Incubated homogenates prepared in parallel were made with 9 vols. of sucrose/phosphate buffer, incubated at 37°C for 60 min and then extracted with chloroform/methanol. Assays of the inhibitor and of fatty acids in the extracts were made as described in Methods.

	Pancreas	
	Intact	Incubated homogenate
Inhibitor (units/mg)	0.024	0.650
Free fatty acids (μg/mg)	1.02	15.32
Free fatty acids (μg/unit)	42.5	23.6
	Percentage composition of free fatty acids in extracts (%)	
14:0	2.6	1.0
16:0	27.8	17.7
16:1	4.4	5.5
18:0	10.1	7.1
18:1	29.6	34.7
18:2	12.2	21.6
20:4	3.1	5.2
Other fatty acids	10.2	7.2

inhibitor (Table VI). Edwards and Ball [25] first described the similarly poor inhibitory action of $C_{18:0}$ compared with $C_{18:1}$ towards succinate dehydrogenase activity. The C_{14}—C_{20} fatty acids made up more than 90% of the fatty acids in the extracts and summation of the inhibitions that could be attributed to each of the individual acids present accounted for 110 and 74% of the inhibitions observed in the extracts of intact and incubated pancreas, respectively. Three fatty acids, $C_{18:1}$, $C_{18:2}$, and $C_{20:4}$, could account for 86% of the observed inhibition of the incubated extract.

Solvent extracts of liver were analyzed in the same way as pancreas. Incubation of liver homogenates before extraction produced an increase of free fatty

TABLE VI

INHIBITION OF PROLINE OXIDASE ACTIVITY BY FREE FATTY ACIDS

Fatty acids were dissolved in light ether which was then evaporated under N_2 gas. The fatty acids were uniformly suspended in water for the determination of the inhibitory activity of proline oxidase.

Free fatty acid	For 50% inhibition (μg)
14:0	38.2
16:0	87.1
16:1	26.1
18:0	2162.1
18:1	21.8
18:2	27.7
20:4	16.6

acids and a parallel increase of the proline oxidase inhibitor. However, the yield was only 0.09 unit of inhibitor/mg liver. The free fatty acids present per unit of inhibitor were 33 μg. This is comparable to the amounts per unit in pancreas extracts (43 and 24 μg in intact and incubated extracts) and to the unit of inhibition by palmitoyl-CoA (55 μg).

Discussion

The results of the present study indicate that the inhibition of proline oxidase by pancreas homogenate is mainly due to its high concentrations of free fatty acids. The properties of the inhibitory activity, i.e., its association with the particulate fraction, the relase of inhibition by albumin in amounts which bind the appropriate amounts of fatty acids [26], and its resistance to heat treatment at 100°C for 10 min, are all consistent with the properties of fatty acids. Furthermore, 85% or more of the inhibitory activity of pancreas homogenate is extractable into chloroform/methanol and virtually all of the recoverable inhibitory activity in this extract is found in the free fatty acid fraction. At the concentrations found in the pancreas extract, free fatty acids themselves have sufficient inhibitory activity towards proline oxidase to account for all the inhibitory activity in that fraction. Fatty acids isolated from liver are equally inhibitory but occur in much lower amounts.

Although there is a large amount of free fatty acids in incubated pancreas homogenates (15 mg/g wet weight of pancreas), there is considerably less (1 mg/g wet weight of pancreas) if the pancreas is first heated or if intact pancreas is directly extracted with chloroform/methanol. The fatty acids are produced in a time-dependent manner by a heat-labile process, presumably by the action of a lipase. Lack of this lipase until shortly after birth could account for the presence of proline oxidase in infantile pancreas homogenates. The profile of free fatty acids in pancreas homogenate, with its excess of unsaturated fatty acids, resembles the pattern of fatty acids found in triglycerides [27]. The dependence upon enzymic hydrolysis as well as the composition of the fatty acids isolated suggests that their real source, and the source of the inhibitory activity in the pancreas homogenates, is primarily from the hydrolysis by lipase of triglycerides. Presumably these come from the fat cells that are a prominent feature of the rat pancreas. Some portion of the inhibition, namely that lost with washing of the chloroform extract (Table III) or after its treatment with cysteine (Fig. 1), may consist of other lipid species for which palmitoyl-CoA may be a model.

Free fatty acids have often been discussed as possible regulatory inhibitors of specific enzymes, and some form of regulation of proline metabolism is necessary because two unidirectional enzymes that form and degrade proline coexist in many tissues [1]. There can be little reason for attributing such a role to fatty acids, since proline oxidase activity can be demonstrated in pancreas slices, and its inhibition occurs only if tissue disintegration allows the free fatty acids to be formed. The inhibitory action of free fatty acids closely parallels that on a variety of enzymes as described by Taketa and Pogell [4] for palmitoyl-CoA; they concluded that it had a nonspecific, detergent-like effect. There do not appear to be common characteristics among the soluble and insoluble

enzymes inhibited by the fatty acids of pancreas (Table II), and this non-specificity for enzymes also argues generally against free fatty acids as enzyme regulators. However, because of this inhibitory potential a variety of enzymes could possibly be underestimated in pancreatic homogenates.

Acknowledgements

This work was supported by U.S. Public Health Service Research Career Award AM 02018-17 and grant AM 00567-26 from the National Institute of Arthritis, Metabolism and Digestive Diseases, Department of Health, Education, and Welfare. We wish to thank Tamsin A. Knox for her technical help, and Mary Anne Lunetta for her skillful preparation of the manuscript.

References

1 Herzfeld, A., Mezl, V.A. and Knox, W.E. (1977) Biochem. J. 166, 95—103
2 Herzfeld, A. and Raper, S.M. (1976) Biochim. Biophys. Acta 428, 600—610
3 Mezl, V.A. and Knox, W.E. (1976) Anal. Biochem. 74, 430—440
4 Taketa, K. and Pogell, B.M. (1966) J. Biol. Chem. 241, 720—726
5 Shonk, C.E. and Boxer, G.E. (1964) Cancer Res. 24, 709—721
6 Strecker, H.J. (1971) Methods Enzymol. 17B, 258—261
7 Herzfeld, A. and Knox, W.E. (1968) J. Biol. Chem. 243, 3327—3332
8 Mason, T.L., Poyton, R.O., Wharton, D.C. and Schatz, G. (1973) J. Biol. Chem. 248, 1346—1354
9 Williamson, J.R. and Corkey, B.E. (1969) Methods Enzymol. 13, 471—473
10 Herzfeld, A. (1972) Enzyme 13, 246—251
11 Williamson, J.R. and Corkey, B.E. (1969) Methods Enzymol. 13, 466—468
12 Laatsch, R.H., Kies, M.W., Gordon, S. and Alvord, E.C. (1962) J. Exp. Med. 115, 777—788
13 Green, D.E. and Ziegler, D.M. (1963) Methods Enzymol. 6, 416—417
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Folch, J., Lees, M. and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 497—509
16 Wadke, M., Brunengraber, H., Lowenstein, J.M., Dolhum, J.J. and Arsenault, G.P. (1973) Biochemistry 12, 2619—2624
17 Patton, G.M. and Lowenstein, J.M. (1979) Biochemistry 18, 3186—3188
18 Knox, W.E., Linder, M. and Friedell, G.H. (1970) Cancer Res. 30, 282—287
19 Herzfeld, A. and Greengard, O. (1977) Cancer Res. 37, 231—238
20 Phang, J.M., Downing, S.J., Smith, R.J. and Yeh, C.C. (1978) Fed. Proc. 37, 1480
21 Kornberg, A. and Pricer, W.E., Jr. (1953) J. Biol. Chem. 204, 329—343
22 Jamieson, J.D. and Palade, G.E. (1967) J. Cell Biol. 34, 577—596
23 Walsh, K.A. (1970) Methods Enzymol. 19, 41—63
24 Greene, L.J., Rigbi, M. and Fackre, D.S. (1966) J. Biol. Chem. 241, 5610—5618
25 Edwards, S.W. and Ball, E.G. (1954) J. Biol. Chem. 209, 619—633
26 McMenamy, R.H. (1965) J. Biol. Chem. 240, 4235—4243
27 Calderon, P., Furnelle, J. and Christophe, J. (1979) Biochim. Biophys. Acta 574, 379—390