Biochimica et Biophysica Acta, 429 (1976) 564—572

BBA 67745

PROPERTIES OF THE MAJOR CARBOXYPEPTIDASE IN THE LARVAE OF THE WEBBING CLOTHES MOTH, *TINEOLA BISSELLIELLA*

COLIN W. WARD

Division of Protein Chemistry, C.S.I.R.O., Parkville (Melbourne), Victoria 3052 (Australia)

(Received August 26th, 1975)

Summary

The larvae of the webbing clothes moth, *Tineola bisselliella* contain two carboxypeptidases (EC 3.4.12-) and one of these has been purified by preparative polyacrylamide gel electrophoresis. Its pH optimum for the hydrolysis of *N*-benzyloxycarbonyl-glycyl-leucine was pH 7.5—7.7 and its molecular weight as judged by gel filtration was 72 000. It is strongly inhibited by diisopropylfluorophosphate, thiol reagents and some metal cations and also by 1 : 10 phenanthroline but not EDTA. K_m and V values for the hydrolysis of 13 *N*-acyl dipeptides were determined. The enzyme has a strong preference for neutral aliphatic amino acid residues and does not hydrolyse C-terminal proline, arginine or lysine. It is a true carboxypeptidase, requiring an L-amino acid in the C-terminal position, with a free carboxyl group and hydrolysing peptide substrates consecutively from the C-terminal end. Dipeptides are cleaved much more slowly than tripeptides or *N*-acyl dipeptides.

Introduction

In a preliminary investigation [1] into the proteinases and peptidases present in the larvae of the webbing clothes moth, *Tineola bisselliella*, considerable carboxypeptidase (EC 3.4.12-) activity against *N*-benzyloxycarbonyl (Z)-Gly-Leu was detected, and this could be fractionated into two well separated peaks by chromatography on DEAE-cellulose at pH 7.7. Acrylamide gel zymograms showed that the first, and smaller, peak contained a single carboxypeptidase band of R_F 0.3, while the second, larger peak contained a single carboxypeptidase band of R_F 0.69.

Abbreviations: Bz, *N*-benzoyl; Diol buffer, 2-amino-2-methyl-1,3-propandiol buffer; Z, *N*-benzyloxycarbonyl.

In this paper the latter enzyme has been purified and some of its properties, including its action on several free and blocked peptide substrates, investigated.

Materials and Methods

Chemicals. Unless otherwise stated, all peptide substrates contained only L-amino acids. All benzoyl and benzyloxycarbonyl peptides were obtained from Schwarz-Mann Research Laboratories.

The peptides Leu-Leu and Leu-Ala, were from Cyclo Chemical Corp. and Leu-Gly was from Calbiochem. Leu-Pro, Pro-Leu, L-Leu-D-Leu, D-Leu-L-Leu, Ala-Leu and Leu-Gly-amide were from Bachem Fine Chemicals. Leu-Ile, Leu-Phe, Leu-Val, Leu-Leu-Leu, Leu-Gly-Leu, Ala-Gly-Leu, Gly-Ala-Leu, Leu-Thr-Gly, Ala-Gly-Phe, Leu-Gly-Val and Ala-Gly-Phe-Gly-Gly were kindly prepared by Dr. F.H.C. Stewart of this laboratory. Bovine serum albumin, L-amino acid oxidase (*Crotalus adamanteus* venom) and horseradish peroxidase were from Sigma Chemical Co. and σ-dianisidine was from B.D.H. Chemical Co.

Enzyme. The starting material used was fraction D2 obtained as previously described [1] and stored in 50% glycerol at −20°C until used.

Enzyme assays. Carboxypeptidase assays were carried as described previously [1].

Enzyme kinetic determinations with peptide substrates. The kinetic parameters K_m and V for the hydrolysis of benzoyl and benzyloxycarbonyl peptides were determined by the method of Lee and Wilson [2] where the instantaneous velocity is replaced by the average velocity over a fixed time period and the initial substrate concentration is replaced by the average substrate concentration during that time interval. Regression analysis of the Lineweaver-Burk plots of $1/\bar{v}$ versus $1/\bar{S}$, $\bar{S}/\bar{v}$ versus $\bar{S}$ and the Eadie-Hofstie plot, $\bar{v}/\bar{S}$ versus $\bar{v}$, were carried out on a GE terminal computer. The K_m and V values from these three regression analyses were averaged. Initial substrate concentrations employed were $0.1 \cdot 10^{-3}$, $0.25 \cdot 10^{-3}$, $0.5 \cdot 10^{-3}$, $1.0 \cdot 10^{-3}$, $2.5 \cdot 10^{-3}$ and $5 \cdot 10^{-3}$ M. Reaction conditions were 37°C, pH 7.7, and incubation time 30 min.

Hydrolysis of peptide substrates. The hydrolysis of peptide substrates was performed at 37°C in 0.05 M 2-amino-2-methyl-1,3-propandiol(Diol)buffer, pH 8.0, with 2.5 mM substrate and 10 µl carboxypeptidase D2a. The extent of hydrolysis after 3 h incubation was monitored qualitatively, by high voltage electrophoresis [3] at pH 3.5 [4] on Whatman 3MM paper and quantitatively by amino acid analysis with a Beckman 120C amino acid analyzer.

Acrylamide gel electrophoresis. Electrophoresis in gel rods and gel slabs (120 mm long, 70 mm wide, 3 mm thick) and the detection of carboxypeptidase bands in gels were as described previously [1]. For preparative slab gels, the Tris/glycine tray buffer was replaced by Tris/borate buffer, pH 8.3 (see ref. 5), the enzyme was located by staining side strips for activity and the enzyme was recovered electrophoretically after macerating the gel through a 16-gauge needle into a special cell fitted with a collecting tube sealed at one end by dialysis membrane. The buffer used for electrophoretic elution was the Tris/borate tray buffer.

Protein determination. Protein was estimated by the method of Lowry et al. [6] with bovine serum albumin as standard.

Results

Final purification

In addition to the single carboxypeptidase band (R_m 0.69), the starting material, fraction D2, contained several aminopeptidases and a small amount of proteinase activity [1]. These contaminants were removed by preparative electrophoresis in 7.5% acrylamide gel slabs. Initially the Tris/glycine buffer system of Davis [5] was used and the glycine (which interferes with the ninhydrin carboxypeptidase assay) removed from the recovered enzyme by dialysis. However, considerable enzyme activity was lost during dialysis and an alternative electrophoretic procedure using Tris/borate tray buffers [5] was adopted.

As shown in Fig. 1, the Tris/borate buffer system gave the same electrophoretic pattern as Tris/glycine and enzyme activity could still be detected readily. When purified on preparative slab gels the material containing carboxypeptidase activity (fraction D2a) gave only a single protein band on acrylamide gels and this coincided with the position of the enzyme activity (Fig. 1).

Properties of carboxypeptidase D2a

pH optimum. As shown in Fig. 2, the pH optimum for the hydrolysis of Z-Gly-Leu by the purified carboxypeptidase was pH 7.5—7.7. Very little activity could be detected below pH 6.0. Phosphate buffers could not be used as they were found to be strongly inhibitory.

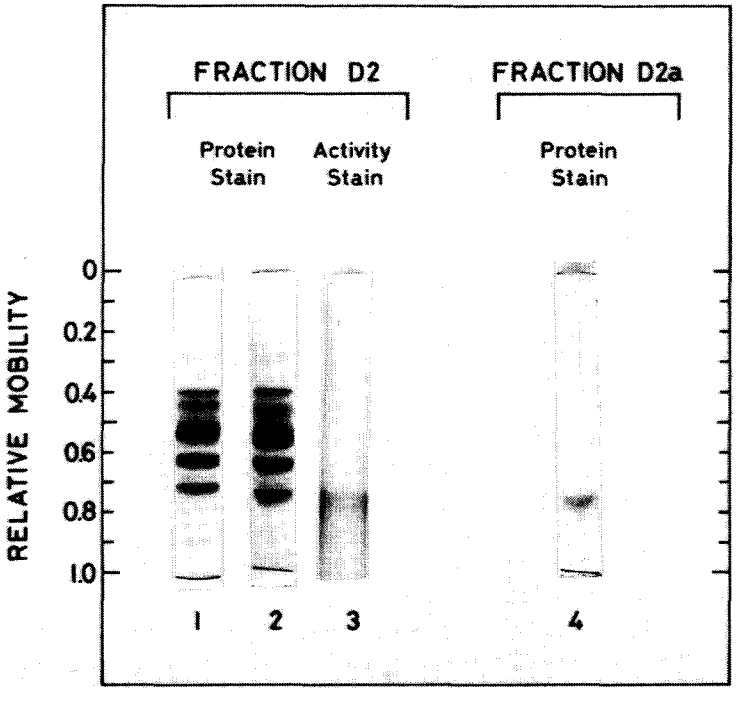

Fig. 1. Protein bands and carboxypeptidase activity bands after acrylamide gel electrophoresis of fractions D2 and D2a. Gels 1 and 3 were run with the Tris/glycine buffer system while gels 2 and 4 were run with the Tris/borate system (see Materials and Methods).

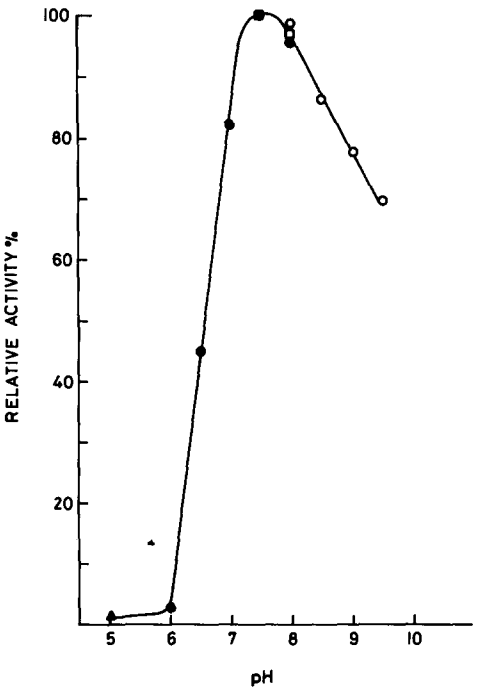

Fig. 2. Effect of pH of Z-Gly-Leu hydrolysis by fraction D2a. Buffers used at 0.01 M were: ▲, sodium acetate/acetic acid; ● *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)/NaOH; □, Tris—HCl; ○, Diol/HCl. Maximum activity was 11.2 μmol Z-Gly-Leu hydrolysed/30 min per ml enzyme.

Inhibitors. As reported previously with crude preparations [1], the purified enzyme was almost completely inhibited by the thiol reagents *p*-chloromercuribenzoate and iodoacetate and by the metal chelator 1 : 10 phenanthroline, but not by EDTA (Table I). It was also strongly inhibited by diisopropylfluorophosphate.

TABLE I

EFFECT OF INHIBITORS AND METAL IONS ON D2a CARBOXYPEPTIDASE ACTIVITY

The enzyme was pre-incubated with inhibitor for 30 min at pH 7.5 and 20°C, then Z-Gly-Leu added (0.001 M) and the residual carboxypeptidase activity determined at 37°C as described previously [1].

Additions	Final concentration (M)	Effect on carboxypeptidase
p-Chloromercuribenzoate	0.0001	100% inhibition
Iodoacetate	0.001	90% inhibition
EDTA	0.001	No effect
1 : 10 phenanthroline	0.001	93% inhibition
DFP	0.001	95% inhibition
$CaCl_2$	0.001	59% inhibition
$MnSO_4$	0.001	31% inhibition
$FeSO_4$	0.001	25% inhibition
$CoCl_3$	0.001	58% inhibition
$ZnCl_2$	0.001	56% inhibition
$CuCl_2$	0.001	94% inhibition
$HgCl_2$	0.001	100% inhibition

TABLE II

KINETIC CONSTANTS FOR HYDROLYSIS OF BENZOYL AND BENZYLOXYCARBONYL PEPTIDES BY CARBOXYPEPTIDASE D2a

V values are expressed relative to the value of 12.5 μmol/30 min per ml enzyme obtained for the hydrolysis of Z-Gly-Leu. Reaction conditions were 37°C, pH 7.7; incubation time, 30 min. Assays were as described [1] and the kinetic parameters determined as outlined in Materials and Methods.

Substrate	K_m (mM)	V (relative %)	V/K_m (relative %)
Z-Gly-Leu	0.42	100	100
Z-Ala-Phe	0.36	63.7	74.6
Z-Gly-Glu	0.24	17.0	29.6
Z-Gly-Tyr	0.38	19.4	21.1
Z-Gly-Phe	0.44	12.0	11.6
Bz-Gly-Gly-Gly	6.03	47.6	3.3
Z-Gly-Asp	0.78	4.5	2.4
Z-Gly-Ala	6.34	26.4	1.7
Z-Gly-Trp	1.54	5.9	1.6
Z-Gly-Ser Z-Gly-Pro Bz-Gly-Lys Bz-Gly-Arg	Not hydrolysed.		

The effect of various metal ions on the purified carboxypeptidase is also shown in Table I. Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{3+} and Zn^{2+} produced partial inhibition while Cu^{2+} and Hg^{2+} produced strong inhibition.

Molecular weight. Carboxypeptidase D2a eluted from a Sephadex G-200 column calibrated with γ-globulin, bovine serum albumin dimer and monomer, ovalbumin, carbonic anhydrase, myoglobin and cytochrome *c* [7] at a position corresponding to a molecular weight of 72 000.

Hydrolysis of benzoyl and benzyloxycarbonyl peptide substrates. The kinetic parameters determined for the hydrolysis of several benzoyl and benzyloxy-

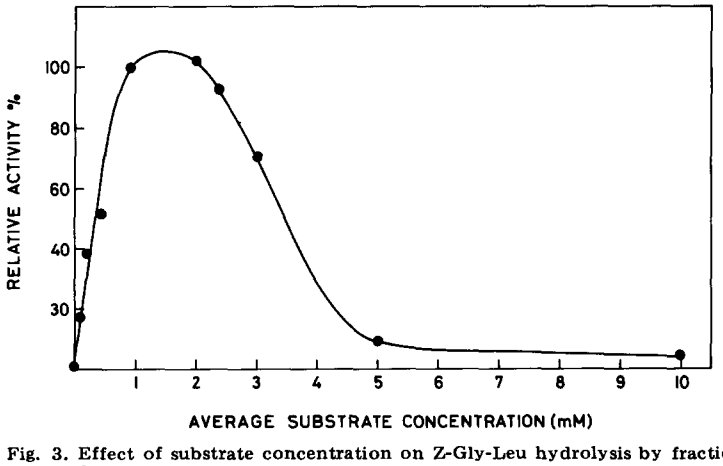

Fig. 3. Effect of substrate concentration on Z-Gly-Leu hydrolysis by fraction D2a. Reaction conditions were 37°C, pH 7.7; incubation time, 30 min. Initial substrate concentrations were $0.1 \cdot 10^{-3}$, $0.25 \cdot 10^{-3}$, $0.5 \cdot 10^{-3}$, $1.0 \cdot 10^{-3}$, $2.5 \cdot 10^{-3}$ and $5 \cdot 10^{-3}$ M. Activity values are expressed relative to that of 11.2 μmol Z-Gly-Leu hydrolysed/30 min per ml enzyme obtained at average substrate concentration of $0.86 \cdot 10^{-3}$ M.

TABLE III

HYDROLYSIS RATES OF PEPTIDE SUBSTRATES BY CARBOXYPEPTIDASE D2a

Values indicated are the relative hydrolysis rates of the C-terminal peptide bond in 3 h at 37°C by carboxypeptidase D2a. The hydrolyses were monitored, by high voltage electrophoresis at pH 3.5 and quantitated by amino acid analysis as described in Materials and Methods.

Dipeptides		Tripeptides		Other peptides	
Leu-Gly	0.8	Leu-Thr-Gly	2.9	Leu-Gly-NH_2	0
Leu-Ala	2.8	Leu-Gly-Val	45.6	Ala-Gly-Phe-Gly-Gly	3.1
Leu-Val	1.0	Leu-Gly-Leu	100		
Leu-Ile	4.3	Gly-Ala-Leu	89.6		
Leu-Leu	6.3	Leu-Leu-Leu	38.1		
L-Leu-D-Leu	0	Ala-Gly-Leu	19.1		
D-Leu-L-Leu	0	Ala-Gly-Phe	3.1		
Leu-Phe	1.3				
Leu-Pro	0				
Pro-Leu	0				
Ala-Leu	3.5				

carbonyl peptide derivatives are shown in Table II. The most favoured substrate was Z-Gly-Leu, while the substrates Z-Gly-Ser, Z-Gly-Pro, *N*-benzoyl(Bz)-Gly-Arg, Bz-Gly-Lys were not hydrolysed at all. This *T. bisselliella* carboxypeptidase exhibited marked substrate inhibition at Z-Gly-Leu concentrations greater than $2 \cdot 10^{-3}$ M (Fig. 3).

Hydrolysis of peptide substrates. Table III shows the relative rates of hydrolysis of several peptide substrates by carboxypeptidase D2a. Tripeptides were hydrolysed much more rapidly than dipeptides. Dipeptide amides and peptide bonds involving proline or D-amino acids were not hydrolysed by this enzyme.

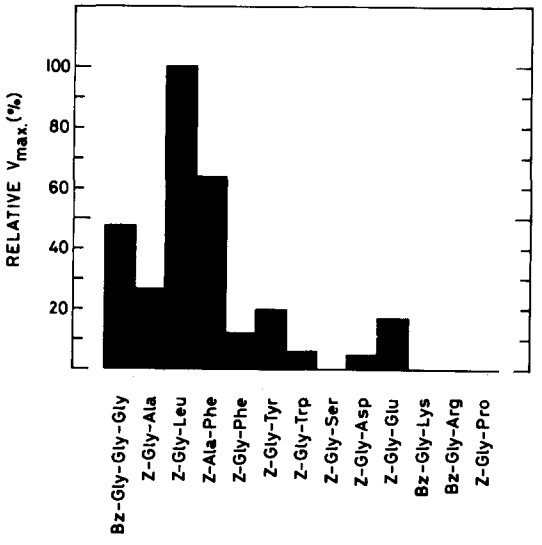

Fig. 4. Relative hydrolysis rates of benzoyl- and benzyloxycarbonyl peptides by *T. bisselliella* carboxypeptidase.

Discussion

In a previous communication [1] the larvae of the webbing clothes moth, *T. bisselliella* were shown to contain a complex mixture of proteolytic enzymes which were associated with their digestive tract. Trypsin-like, chymotrypsin-like and metal chelator-sensitive proteinases were present as well as several aminopeptidases and two carboxypeptidase type activities. Recent reports have dealt with the purification and properties of the aminopeptidases [8—10], the trypsin-like proteinases [11], and the metal chelator-sensitive proteinases [12,13]. In this report the major carboxypeptidase from these larvae has been further purified and some of its properties compared with those of carboxypeptidases from other sources.

Its pH optimum of 7.5—7.7 is very similar to the values of 7.4—7.8 reported for pancreatic carboxypeptidase A [14] and 7.8—8.2 for carboxypeptidase B [15]. It further resembles carboxypeptidases from other sources in its strong inhibition by phosphate ions [14,16,17].

Its molecular weight of 72 000 is quite different to that of 34 000 reported for pancreatic carboxypeptidases A [18] and B [19] and for shrimp hepatopancreas carboxypeptidases [20], but more closely resembles the carboxypeptidases from yeast [21], mould [22], and plants [23—26].

Like the carboxypeptidases from other sources its response to inhibitors is complicated. It appears to be a serine type carboxypeptidase since it is strongly inhibited by low concentrations of diisopropylfluorophosphate. It also appears to have a requirement for a free sulphydryl group since it is strongly inhibited by *p*-chloromercuribenzoate, iodoacetate and several metal ions [27]. The nature of this enzyme is further complicated by its strong inhibition by 1 : 10 phenanthroline but not by EDTA. The serine carboxypeptidases from yeast [21] and barley [24] show dual inhibition by diisopropylfluorophosphate and thiol reagents, while those from cotton seed [26] and French bean seed [28] are only inhibited by diisopropylfluorophosphate. These serine type carboxypeptidases are quite different from the vertebrate pancreatic carboxypeptidases, which are zinc metalloenzymes, inhibited by thiol reagents [14] and the metal chelator 1 : 10 phenanthroline but not by EDTA [19,29].

The kinetic parameters obtained for the hydrolysis of several benzoyl- and benzyloxycarbonyl dipeptides indicate some similarity between this *T. bisselliella* enzyme and pancreatic carboxypeptidase A. Peptides with C-terminal lysine or arginine were not cleaved [30] and neither were those with C-terminal proline [31]. Furthermore, extensive substrate inhibition (Fig. 3) was found for the *T. bisselliella* carboxypeptidase as previously reported for carboxypeptidase A [32,33]. However, the insect enzyme differs from carboxypeptidase A in its relative rates of hydrolysis of the various benzoyl- and benzyloxycarbonyl peptides. Those with C-terminal leucine and to a lesser extent alanine or glycine were cleaved more rapidly than those with C-terminal aromatic amino acids. This is in direct contrast to the action of carboxypeptidase A [31,32,34,35]. Substitution of the alanyl group for glycyl in the penultimate C-terminal position produced a 5-fold increase in the rate of release of C-terminal phenylalanine by *T. bisselliella* carboxypeptidase but only a slight decrease in hydrolysis by carboxypeptidase A [36]. A further difference between the two enzymes

is the somewhat lower K_m values obtained for the *N*-acyl dipeptides with the *T. bisselliella* carboxypeptidase.

Additional information on the specificity requirements of the insect carboxypeptidase was obtained with the free peptide substrates (Table III). Like carboxypeptidase A, it requires an L-amino acid in the C-terminal position [31, 37] with a free carboxyl group [34]. Dipeptides, which have a free amino group, are hydrolysed much more slowly than tripeptides or *N*-acyl dipeptides [34] presumably because of the electrostatic effect of the positive charge on the amino group [38].

The results reported in this paper show that this enzyme from *T. bisselliella* larvae is a true carboxypeptidase which hydrolyses peptide substrates consecutively from the C-terminal end. It resembles pancreatic carboxypeptidase A in its pH optimum, some specificity requirements and kinetic characteristics but not in its molecular weight or the nature of its active site. In these latter respects it resembles the serine carboxypeptidases from some micro-organisms and plants.

Studies are in progress to characterize the second carboxypeptidase from *T. bisselliella* larvae [1] to see if it is analogous to carboxypeptidase B [39]. Few carboxypeptidases have been reported in invertebrates [40—44] and the only well characterized enzymes are the carboxypeptidases A and B from shrimp hepatopancreas [20] and the carboxypeptidase B from starfish pyloric caeca [45]. These enzymes were zinc metalloproteins, very similar in their properties to the vertebrate carboxypeptidases A and B.

Acknowledgements

I wish to thank Mr. W.J. Youl for his technical assistance and Dr. C.M. Roxburgh for the amino acid analysis.

References

1 Ward, C.W. (1975) Aust. J. Biol. Sci. 28, 1—23
2 Lee, H.-J. and Wilson, I.B. (1971) Biochim. Biophys. Acta 242, 519—522
3 Bennet, J.C. (1967) Methods Enzymol. 11, 330—339
4 Naughton, M.A., Sanger, F., Hartley, B.S. and Shaw, D.D. (1960) Biochem. J. 77, 149—163
5 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
7 Andrews, P. (1965) Biochem. J. 96, 595—606
8 Ward, C.W. (1975) Int. J. Biochem. 6, 765—768
9 Ward, C.W. (1975) Biochim. Biophys. Acta 410, 361—369
10 Ward, C.W. (1975) Aust. J. Biol. Sci. 28, 447—455
11 Ward, C.W. (1975) Biochim. Biophys. Acta 391, 201—211
12 Ward, C.W. (1975) Biochim. Biophys. Acta 384, 215—227
13 Ward, C.W. (1975) Aust. J. Biol. Sci. 28, 439—445
14 Smith, E.L. and Hanson, H.T. (1949) J. Biol. Chem. 179, 803—813
15 Folk, J.E. and Gladner, J.A. (1958) J. Biol. Chem. 231, 379—391
16 Wolff, E.C., Schirmer, E.W. and Folk, J.E. (1962) J. Biol. Chem. 237, 3094—3099
17 Zuber, H. (1964) Nature 201, 613
18 Bradshaw, R.A., Ericsson, L.H., Walsh, K.A. and Neurath, H. (1969) Proc. Natl. Acad. Sci. U.S. 63, 1389—1394
19 Folk, J.E., Piez, K.A., Carroll, W.R. and Gladner, J.A. (1960) J. Biol. Chem. 235, 2272—2277
20 Gates, B.J. and Travis, J. (1973) Biochemistry 12, 1867—1874
21 Hayashi, R. and Hata, T. (1972) Biochim. Biophys. Acta 263, 673—679

22 Jones, S.R. and Hofmann, T. (1972) Can. J. Biochem. 50, 1297—1310
23 Wells, J.R.E. (1968) Biochim. Biophys. Acta 167, 388—398
24 Visuri, K., Mikola, J. and Enari, T-M. (1969) Eur. J. Biochem. 7, 193—199
25 Sprössler, B., Heilmann, H-D., Grampp, E. and Uhlig, H. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1524—1530
26 Ihle, J.N. and Dure, L.S. (1972) J. Biol. Chem. 247, 5041—5047
27 Vallee, B.L. and Wacker, W.E.C. (1970) The Proteins (Neurath, H., ed.), 2nd edn., Vol. 5, pp. 129—146, Academic Press, New York
28 Shaw, D.C. and Wells, J.R.E. (1972) Biochem. J. 128, 229—235
29 Felber, J.P., Coombs, T.L. and Vallee, B.L. (1962) Biochemistry 1, 231—238
30 Pétra, P.H. (1970) Methods Enzymol. 19, 460—503
31 Stahmann, M.A., Fruton, J.S. and Bergmann, M. (1946) J. Biol. Chem. 164, 753—760
32 Lumry, R., Smith, E.L. and Glantz, R.R. (1951) J. Am. Chem. Soc. 73, 4330—4340
33 McClure, W.O., Neurath, H. and Walsh, K.A. (1964) Biochemistry 3, 1897—1901
34 Hofmann, K. and Bergmann, M. (1940) J. Biol. Chem. 134, 225—235
35 Smith, E.L. (1948) J. Biol. Chem. 175, 39—47
36 Bergmann, M. and Fruton, J.S. (1942) J. Biol. Chem. 145, 247—252
37 Hanson, H.T. and Smith, E.L. (1949) J. Biol. Chem. 179, 815—818
38 Neurath, H. (1960) The Enzymes (Boyer, P.D., Lardy, H. and Myrbäck, K., eds.), 2nd edn., Vol. 4, pp. 11—36, Academic Press, New York
39 Wintersberger, E., Cox, D.J. and Neurath, H. (1962) Biochemistry 1, 1069—1078
40 De Villez, E.J. (1968) Am. Zool. 8, 541
41 Camacho, Z., Brown, J.R. and Kitto, G.B. (1970) J. Biol. Chem. 245, 3964—3972
42 Reid, R.G.B. and Rauchert, K. (1970) Comp. Biochem. Physiol. 35, 689—695
43 Winter, W.P. and Neurath, H. (1970) Biochemistry 9, 4673—4679
44 Elyakova, L.A. and Kozlovskaya, E.P. (1975) Comp. Biochem. Physiol. 50B, 249—253
45 Ferrell, R.E., Camacho, Z. and Kitto, G.B. (1975) Biochim. Biophys. Acta 386, 260—269