Proc. Natl. Acad. Sci. USA
Vol. 78, No. 4, pp. 2155–2158, April 1981
Biochemistry

Enzymatic reduction of protein-bound methionine sulfoxide

(oxidation of methionine residues/inactive proteins/ribosomal protein L12)

NATHAN BROT, LAWRENCE WEISSBACH *, JEAN WERTH†, AND HERBERT WEISSBACH

Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Communicated by B. L. Horecker, December 24, 1980

ABSTRACT **An enzyme that catalyzes the reduction of methionine sulfoxide residues in ribosomal protein L12 has been partially purified from *Escherichia coli* extracts. Methionine sulfoxide present in oxidized [Met]enkephalin is also reduced by the purified enzyme. The enzyme is different from a previously reported *E. coli* enzyme that catalyzes the reduction of methionine sulfoxide to methionine [Ejiri, S. I., Weissbach, H. & Brot, N. (1980) *Anal. Biochem.* 102, 393–398]. Extracts of rat tissues, *Euglena gracilis*, *Tetrahymena pyriformis*, HeLa cells, and spinach also can catalyze the reduction of methionine sulfoxide residues in protein.**

In vivo experiments have suggested the presence of an enzyme system in *Escherichia coli* that converts methionine sulfoxide (MetSO) to methionine (1, 2). We have recently reported the presence of an enzyme in extracts of *E. coli* that catalyzes the reduction of MetSO to methionine (3, 4). A similar reaction was described in yeast by Black *et al.* (5) who showed that three protein factors were required. These factors were later shown to be thioredoxin, thioredoxin reductase, and the enzyme directly responsible for the reduction of MetSO (6). A MetSO-reducing system has also been shown to be present in animal tissues and plants (7–9). Our previous studies on the purification of the enzyme system from *E. coli* showed that dithiothreitol could replace reduced thioredoxin, which simplified the assay considerably (4).

Because high levels of MetSO would not be expected to occur in tissues, it was postulated that a MetSO-reducing system in cells might be present to reduce MetSO that accumulated in proteins (3, 4). There are now many examples in which proteins lose their biological activity when their methionine residues are oxidized to the sulfoxide (10–23). This may occur *in vivo* because oxidizing agents such as peroxides, hydroxyl radicals, and superoxides are constantly being formed. Of special interest is the situation in the lens. One of the major differences between normal and cataractous lens is the relatively large amount of MetSO in the cataractous lens protein (24, 25).

The ability of cells to prevent the accumulation of inactive proteins, due to oxidation of methionine, may be crucial for normal function. The presence of an enzymatic system to reduce protein-bound MetSO provides one mechanism for protection against such damage. One of the difficulties in detecting such an enzyme system has been the lack of a suitable assay. Previous studies with *E. coli* ribosomal protein L12 had shown that chemical oxidation of the methionine residues in the protein resulted in the loss of biological activity (10). Thus, oxidized L12 (MetSO-L12) could no longer bind to 50S ribosomal subunits depleted of L12, interact with L10 to form an L12·L10 complex, or be acetylated on the amino-terminal serine to form L7. This last reaction provided a simple assay to measure the conversion of MetSO-L12 to L12.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

In the work reported here, an enzyme has been partially purified from *E. coli* extracts that catalyzes the reduction of MetSO-L12 to L12 and is distinct from the MetSO reductase described previously (3, 4). A similar enzymatic activity has also been detected in plant and animal cells.

MATERIALS AND METHODS

Ribosomal protein L12 was isolated and purified from *E. coli* ribosomes as described (26). The methionine residues in L12 were oxidized with *N*-chlorosuccinamide by using the procedure of Shechter *et al.* (27). The oxidized L12 was dialyzed for 16 hr against 10 mM Tris·HCl, pH 7.9/10 mM $MgCl_2$/10 mM NH_4Cl (buffer A). Oxidized [Met]enkephalin ([MetSO]enkephalin) was kindly supplied by S. Paabo and S. Stein of this institute. The methionine residue in [*tyrosyl*-3,5-^{3}H]enkephalin (5-L-methionine) was oxidized to MetSO by incubating the enkephalin in a mixture containing 50 μl of dimethyl sulfoxide, 250 μl of 12 M HCl, and 500 μl of glacial acetic acid in a final volume of 1 ml. After 15 min at 23°C, the reaction mixture was dried by evaporation under reduced pressure and dissolved in 740 μl of 4 M urea/1 M formic acid/0.8 M pyridine. The oxidized [Met]enkephalin was purified by high-performance liquid chromatography on an RP-8 column as described by Lewis *et al.* (28). Thioredoxin and thioredoxin reductase were kind gifts of Arne Holmgren (Karolinska Institutet, Stockholm).

Assay for MetSO-L12 Reductase. The assay for the reduction of MetSO-L12 to L12 is based on the ability of L12-transacetylase to specifically acetylate the amino-terminal serine of L12 to form acetyl-L12, (i.e., L7) (29, 30). A two-step incubation was used. The mixture for the first reaction (i.e., reduction) contained (in a total volume of 15 μl) 33 mM Tris·HCl (pH 7.4), 13 mM $MgCl_2$, 275 pmol of MetSO-L12, 13 mM dithiothreitol, and enzyme (see below). After a 60-min incubation at 37°C, the reaction mixtures were placed on ice, to each reaction mixture were added 2 μmol of sodium arsenite, 1.2 μg of partially purified L12-transacetylase (see below), and [^{3}H]acetyl-CoA (935 pmol; 237 cpm/pmol), and the volume was brought to 50 μl. The second incubation (acetylation reaction) was for 5 min at 37°C, after which the reaction mixtures were diluted with 3 ml of cold buffer A and filtered through a nitrocellulose filter. The filters were washed with 10 ml of this same buffer and dissolved in scintillation fluid, and the radioactivity was measured. Under these conditions, L12 and acetyl-L12 are retained quantitatively by the nitrocellulose filter. The results are reported as pmol of acetyl-L12 formed under these conditions.

Assay for MetSO Reductase. The assay for the conversion of MetSO to methionine has been described (4). Briefly, the

Abbreviations: MetSO, methionine sulfoxide; MetSO-L12, oxidized ribosomal protein L12.

* Present address: Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL 32610.
† Visiting Scientist from William Paterson College, Wayne, NJ 07470.

assay involves the acylation of $tRNA^{Met}$ with [^{35}S]methionine formed as a result of the enzymatic reduction of [^{35}S]MetSO. The assay can detect the formation of <1 pmol of methionine.

Assay for Reduction of [MetSO]Enkephalin. The reaction mixture for assaying the reduction of [MetSO]enkephalin contained (in a total volume of 15 μl) 33 mM Tris·HCl, (pH 7.4), 13 mM $MgCl_2$, 13 mM dithiothreitol, 204 pmol of [[^{3}H]MetSO]enkephalin, and 3.2 μg of the partially purified *E. coli* MetSO-L12 reductase (see below). The reaction mixture was incubated for 60 min at 37°C and then placed on ice; 1 mmol of [Met]enkephalin was added and the volume was brought to 0.5 ml with H_2O. An aliquot was applied to an RP-8 column and the column was eluted with a 0–20% *n*-propanol gradient as described (28). Fractions were collected and assayed for radioactivity. Two peaks of radioactivity were observed, corresponding to [Met]enkephalin and [MetSO]enkephalin, and the amount of radioactivity that eluted with the [Met]enkephalin peak was used to calculate the extent of reduction of [MetSO]enkephalin.

Purification of L12-Transacetylase. Five hundred grams of *E. coli* Q13 was suspended in an equal volume of 10 mM Tris acetate, pH 7.8/14 mM Mg acetate/50 mM NH_4Cl/6 mM 2-mercaptoethanol and disrupted in a Manton Gaulin homogenizer. The suspension was centrifuged at 30,000 × *g* and the supernatant solution was fractionated with $(NH_4)_2SO_4$. The protein (15.0 g) precipitating between 35% and 60% $(NH_4)_2SO_4$ saturation was dissolved in buffer B [10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/50 mM KCl/1 mM 2-mercaptoethanol/10% (vol/vol) glycerol] and dialyzed overnight against buffer B. The protein solution was then chromatographed on a DE-52 column (5 × 50 cm) equilibrated with buffer B. The protein was eluted with a 100–400 mM KCl gradient in buffer B. The enzyme activity eluted at about 230 mM KCl, and these fractions were pooled (8.0 g) and concentrated by pressure filtration, and aliquots were chromatographed on Sephadex G-100 columns (5 × 100 cm) that had been equilibrated with 10 mM Tris·HCl, pH 7.5/1 mM $MgCl_2$/1 mM 2-mercaptoethanol/10% glycerol.

The fractions containing the enzyme activity were pooled (750 mg), concentrated, and applied to a hydroxyapatite column (2 × 15 cm) equilibrated with 25 mM KPO_4, pH 6.9/1 mM 2-mercaptoethanol/10% glycerol. The column was eluted with a 25–250 mM KPO_4 gradient (pH 6.9) containing 1 mM 2-mercaptoethanol and 10% glycerol, and the fractions containing enzymatic activity were pooled and concentrated (72 mg). This procedure yielded a 225-fold purification of the enzyme. Under standard conditions of assay (29, 30), this fraction acetylated 46 nmol of L12 per mg of protein in 5 min. The conversion of L12 to acetyl-L12 catalyzed by this enzyme was linear with L12 concentration, and MetSO-L12 was not a substrate for the enzyme (Fig. 1). These properties are essential in order to use the enzymatic acetylation of L12 as an assay for MetSO-L12 reduction. It is also seen in Fig. 1 that, at all concentrations of L12, only one-third of the protein was acetylated.

Purification of MetSO-L12 Reductase. Two hundred grams of *E. coli* Z^{19} cells were grown in L broth to midlogarithmic phase, harvested, suspended in 300 ml of 10 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/10 mM NH_4Cl/1 mM 2-mercaptoethanol (buffer C), and disrupted by sonication. The suspension was centrifuged at 30,000 × *g* and the supernatant was brought to 80% saturation with $(NH_4)_2SO_4$. The precipitate was centrifuged and suspended in 20 mM Tris, pH 7.4/25 mM KCl/2 mM 2-mercaptoethanol/15% glycerol (buffer D) and dialyzed against this buffer. This material (4.5 g) was applied to a DE-52 column (5 × 25 cm) equilibrated with buffer D, and the column was eluted with a 25–500 mM KCl gradient in buffer D. The fractions containing the enzymatic activity were pooled (the enzyme eluted at about 230 mM KCl) and brought to 80% saturation with $(NH_4)_2SO_4$, and the precipitate (1938 mg) was dissolved in buffer C and chromatographed on an Ultrogel AcA-44 column (2.5 × 100 cm) equilibrated with buffer C. The column was eluted with buffer C and the fractions containing enzymatic activity were pooled and concentrated by pressure dialysis. This material (151 mg) was then loaded onto a DEAE-Sephadex column equilibrated with buffer D and the column was eluted with a 25–500 mM KCl gradient in buffer D.

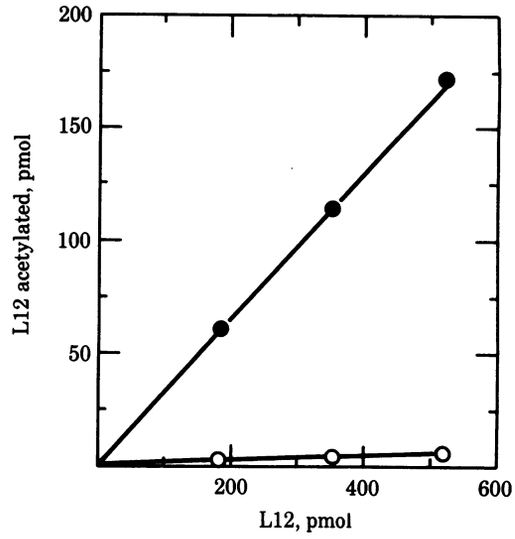

FIG. 1. Effect of L12 and MetSO-L12 concentration on the acetylation reaction. The reaction mixtures contained 1.2 μg of purified L12-transacetylase, and the incubations were at 37°C for 5 min.

The active fractions were pooled and concentrated by pressure filtration (36 mg). In the two-step assay described above, 1 μg of the purified protein catalyzed the reduction of an amount of MetSO-L12 such that 14–19 pmol of acetyl-L12 was formed in the acetylation reaction with L12 transacetylase. It was estimated that the enzyme was purified about 20-fold by this procedure. An exact value could not be obtained because at low protein levels there was not a linear relationship between protein concentration and reductase activity, especially with the less-pure fractions. The partially purified MetSO-L12 reductase preparation did not contain any L12 transacetylase activity.

Preparation of Tissue and Cell Extracts. Three-month-old male rats (150 g) were killed by cervical dislocation, and the various organs were rapidly removed and placed on ice. Each organ was suspended in buffer B (1 g/ml) and homogenized in a Tekmar tissue homogenizer.

Euglena gracilis was grown in the dark at 27°C in a heterotrophic medium as described (31). After the cells had reached a density of 0.8 × 10^5 cells per ml, they were exposed to about 400 footcandles of fluorescent light for 48 hr, harvested, suspended in buffer B, and sonicated.

Tetrahymena pyriformis was grown at 30°C as described (32) and harvested at 1.2 × 10^5 cells per ml. The cells were suspended in buffer B and disrupted by sonication.

HeLa S-3 cells were grown at 37°C to a density of 1 × 10^8 cells/ml in F-13 medium (GIBCO), harvested, suspended in an equal volume of buffer B, and disrupted by sonication.

Spinach was obtained from a local purveyor; its leaves dissected free of the stalks and homogenized in 3 vol of buffer B in a Waring Blendor.

An S-30 extract was prepared from all of the above broken cell extracts by centrifugation at 30,000 × *g* for 20 min. The supernatant fraction was used as the source of the enzyme, and

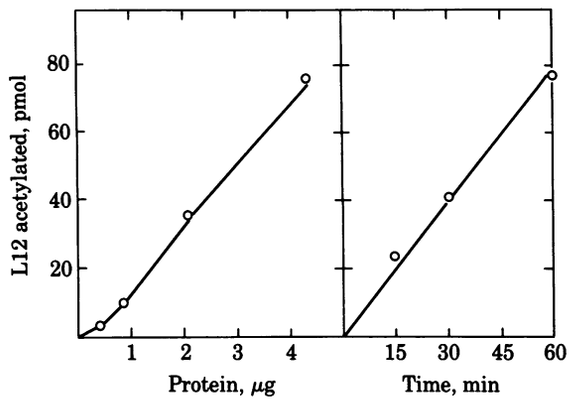

FIG. 2. Effect of protein concentration (*Left*) and time (*Right*) on the reduction of MetSO-L12. (*Left*) Incubation was for 60 min. (*Right*) The protein content was 4.3 μg.

incubations were for 60 min as described above for the assay of the *E. coli* MetSO-L12 reductase.

RESULTS

Reduction of MetSO-L12 to L12. It was noted previously that purified preparations of the *E. coli* enzyme that catalyzed the reduction of free MetSO to methionine did not reduce MetSO-L12 to L12 (4). Because crude *E. coli* extracts had the latter activity, attempts were made to purify this protein(s). The assay was based on the ability of L12 to be acetylated enzymatically compared to MetSO-L12. A partially purified preparation that catalyzed the reduction of MetSO-L12 to L12 was obtained by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose, Ultrogel AcA-44 and DEAE-Sephadex. With the purified enzyme prepration, the reaction was proportional to protein concentration >1 μg per reaction mixture and was linear for 60 min of incubation (Fig. 2).

The reaction was dependent on the enzyme (MetSO-L12 reductase), MetSO-L12, and dithiothreitol (Table 1). Optimal amounts of dithiothreitol were used and could not be replaced by 2-mercaptoethanol. The dithiol appears to have substituted for the normal reductant (i.e., thioredoxin). When the dithiothreitol was replaced by TPNH, thioredoxin, and thioredoxin reductase, there was a large increase in the amount of acetyl-L12 formed.

The highly purified enzyme that catalyzed the reduction of free MetSO to methionine could not catalyze the reduction of MetSO-L12 (4). In a similar manner, the partially purified fraction that catalyzed the reduction of MetSO-L12 had little activity against free MetSO. The enzymatic activities could be separated by Ultrogel chromatography (Fig. 3). The molecular weight of the MetSO-L12 reductase was estimated to be between 18,000 and 20,000 from its profile of elution from the Ultrogel column. In addition, the reduction of MetSO-L12 to L12 was not significantly inhibited by a large excess of MetSO. At 0.1 mM MetSO (about 60 times the concentration of MetSO-L12 used), there was less than 20% inhibition.

Table 1. Requirements for reduction of MetSO-L12

System	Acetyl-L12 formed, pmol
Complete	16.6
Lacking:	
Enzyme	1.3
MetSO-L12	0
Dithiothreitol	3.7
Dithiothreitol; 2-mercaptoethanol added	1.0
Dithiothreitol; TPNH, thioredoxin, and thioredoxin reductase added	52.7

Where indicated, the incubations contained 1 μg of MetSO-L12 reductase protein, 1.4 μg of thioredoxin, 3 μg of thioredoxin reductase, and 50 nmol of TPNH.

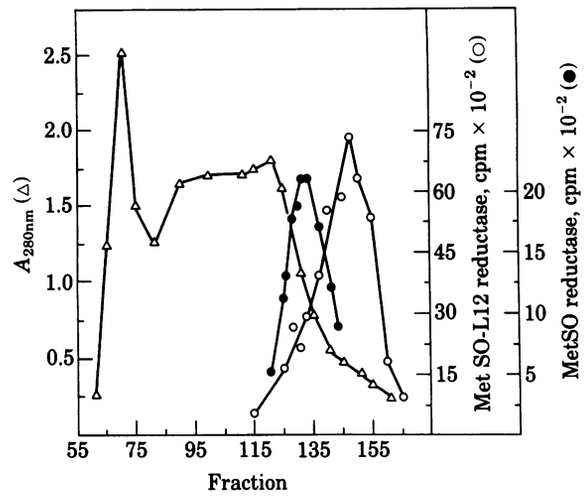

FIG. 3. Separation of MetSO-reducing and MetSO-L12 reducing activities by chromatography of a partially purified preparation of MetSO-L12 reductase on an Ultrogel AcA-44 column.

[MetSO]Enkephalin, which has the sequence Tyr-Gly-Gly-Phe-MetSO, is also a substrate for the enzyme, and, in the presence of dithiothreitol, the oxidized pentapeptide was reduced to [Met]enkephalin (Table 2). Although this reaction was stimulated by dithiothreitol, the reason for the lack of a more complete dependency is not clear.

Preliminary Studies on Distribution of the Enzyme. The activity that reduces MetSO-L12 to L12 has been detected in various cells. Cell-free S-30 extracts were prepared from *Euglena gracilis*, *Tetrahymena pyriformis*, spinach, HeLa cells, and various rat tissues. In all cases, significant reduction of MetSO-L12 was evident (Table 3). However, because of the possible presence of activators or inhibitors in the extracts, and the inability to obtain a linear relationship between activity and amount of extract, the values in Table 3 should be used only for qualitative comparison.

DISCUSSION

The present study describes an enzyme(s) in *E. coli* extracts that can catalyze the reduction of MetSO residues in ribosomal protein L12 to methionine. MetSO-L12 was used as substrate in the present studies because the L12 formed could be acetylated enzymatically and the amount of acetyl-L12 could be determined. Ideally, the enzyme activity should be reported in terms of MetSO residues in the protein that are reduced. Such a value can be deduced from the present experiments if one takes into

Table 2. Reduction of [MetSO]enkephalin by *E. coli* MetSO-L12 reductase

System	[Met]enkephalin, pmol
Complete	32
Lacking:	
Enzyme	2
Dithiothreitol	15

Table 3. Activity of MetSO-L12 reductase, in crude extracts

Source	Acetyl-L12 formed, pmol/mg protein
E. coli	850
Euglena gracilis	2600
Tetrahymena pyriformis	150
Spinach	650
HeLa	330
Liver (rat)	960
Kidney (rat)	923
Heart (rat)	710
Lung (rat)	359
Brain (rat)	181

account the fact that the L12 transacetylase used in the assay only acetylates about one-third of the substrate (Fig. 1) and if the assumption is made that all three methionine residues in MetSO-L12 must be reduced to restore biological activity (10). Because an average of 16 pmol of acetyl-L12 was formed per μg of reductase, this would represent the reduction of 144 pmol of MetSO in the protein.

In addition, it was found that [MetSO]enkephalin can serve as substrate for this enzyme system. Because MetSO is the carboxyl-terminal amino acid in [MetSO]enkephalin, these experiments show that the MetSO residue does not have to occupy an internal position in the protein (or peptide) to be reduced. These results also indicate that the *E. coli* enzyme is not specific for L12.

Previously, we described a similar reaction in *E. coli* that catalyzes the reduction of free MetSO (3, 4). Although this enzyme activity has also been found in yeast (5) and plant (9) and animal (7, 8) tissues, the need for the enzyme remains obscure. The enzyme activity that catalyzes the reduction of MetSO-L12, and perhaps MetSO residues in other proteins, may have a more important physiological function. It is known that a wide variety of oxidizing agents produced in tissues can oxidize protein-bound methionine. Although the deleterious effect of these oxidizing agents would be expected to be neutralized by various cellular enzymes (e.g., catalase, peroxidases, and superoxide dismutase), it is possible that the efficiency of these systems is not sufficient to preclude some oxidation of methionine residues in proteins. For proteins with slow turnover, this oxidation could be crucial if the methionine residues were involved in the protein function. A large number of proteins, including L12, are known to lose biological activity upon oxidation of their methionine residues (10–23).

The results in Table 3 suggest that this activity is widely distributed in nature, and one wonders whether there is a relationship between the level of the MetSO protein reductase activity in various tissues and the presence of a pathological condition. It is known, for example, that α-1 proteinase inhibitor, which is the major serine endopeptidase inhibitor in plasma, is inactivated when specific methionine residues are oxidized (20, 21). This inhibitor is thought to defend against lung tissue proteolysis because there is evidence that emphysema results from a decrease in its activity and the resulting increase in proteolytic activity (21, 33, 34). In addition, it will be of special interest to investigate the cataractous lens proteins. Preliminary results on calf and human lens indicate the presence of an activity that can reduce MetSO-L12 (unpublished data). It will be important to determine whether the activity of this enzyme in lens and lung tissue can be correlated with pathological changes.

The authors express their appreciation to Drs. Svante Paabo and Stanley Stein for performing the enkephalin assays.

1. Lampen, J. O., Jones, M. J. & Perkins, A. B. (1947) *Arch. Biochem. Biophys.* **13**, 33–45.
2. Sourkes, T. L. & Trano, Y. (1953) *Arch. Biochem. Biophys.* **42**, 321–326.
3. Ejiri, S. I., Weissbach, H. & Brot, N. (1979) *J. Bacteriol.* **139**, 161–164.
4. Ejiri, S. I., Weissbach, H. & Brot, N. (1980) *Anal. Biochem.* **102**, 393–398.
5. Black, S., Harte, E. M., Hudson, B. & Wartofsky, L. (1960) *J. Biol. Chem.* **235**, 2910–2916.
6. Porqué, P. G., Baldesten, A. & Reichard, P. (1970) *J. Biol. Chem.* **245**, 2371–2374.
7. Ejiri, S. I., Weissbach, H. & Brot, N. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 829.
8. Aymard, C., Seyer, L. & Cheftel, J. C. (1979) *Agric. Biol. Chem.* **43**, 1869–1872.
9. Doney, R. C. & Thompson, J. F. (1966) *Biochim. Biophys. Acta* **124**, 39–49.
10. Caldwell, P., Luk, D. C., Weissbach, H. & Brot, N. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5349–5352.
11. Carlsson, F. H. H. & Louw, A. I. (1978) *Biochim. Biophys. Acta* **534**, 322–330.
12. Dedman, M. L., Farmer, T. H. & Morris, C. J. O. R. (1961) *Biochem. J.* **78**, 348–352.
13. Jori, G., Galiazoo, G., Marzotto, A. & Scoffone, E. (1968) *Biochim. Biophys. Acta* **154**, 1–9.
14. Jori, G., Galiazzo, G., Marzotto, A. & Scoffone, E. (1968) *J. Biol. Chem.* **243**, 4272–4278.
15. Kido, K. & Kassell, B. (1975) *Biochemistry* **14**, 631–635.
16. Koteliansky, V. E., Domogetsky, S. P. & Gudkov, A. T. (1978) *Eur. J. Biochem.* **90**, 319–323.
17. Neumann, N. P., Moore, S. & Stein, W. H. (1962) *Biochemistry* **1**, 68–75.
18. Ray, W. J., Jr., Latham, H. G., Jr., Katsoulis, M. & Kosland, D. E., Jr. (1960) *J. Am. Chem. Soc.* **82**, 4743–4744.
19. Schachter, H. & Dixon, G. H. (1962) *Biochem. Biophys. Res. Commun.* **9**, 132–137.
20. Del Mar, E. G., Brodrick, J. W., Geokas, M. C. & Largman, C. (1979) *Biochem. Biophys. Res. Commun.* **82**, 346–350.
21. Matheson, N. R., Wong, P. S. & Travis, J. (1979) *Biochem. Biophys. Res. Commun.* **88**, 402–409.
22. Walsh, M. & Stevens, F. C. (1977) *Biochemistry* **16**, 2742–2749.
23. Wolff, J., Cook, G. H., Goldhammer, A. R. & Berkowitz, S. A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3841–3844.
24. Truscott, R. J. W. & Augusteyn, R. C. (1977) *Biochim. Biophys. Acta* **492**, 43–52.
25. Garner, M. H. & Spector, A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1274–1277.
26. Brot, N., Marcel, R., Yamasaki, E. & Weissbach, H. (1973) *J. Biol. Chem.* **248**, 6952–6956.
27. Shechter, Y., Burstein, Y. & Patchornik, A. (1975) *Biochemistry* **14**, 4497–4503.
28. Lewis, R. V., Stein, S. & Udenfriend, S. (1979) *Int. J. Peptide Protein Res.* **13**, 493–497.
29. Brot, N. & Weissbach, H. (1972) *Biochem. Biophys. Res. Commun.* **49**, 673–679.
30. Brot, N., Marcel, R., Cupp, L. & Weissbach, H. (1973) *Arch. Biochem. Biophys.* **155**, 475–477.
31. Fox, L., Erion, J., Tarnowski, J., Spremulli, L., Brot, N. & Weissbach, H. (1980) *J. Biol. Chem.* **255**, 6018–6019.
32. Campbell, J. M. & Weissbach, H. (1980) *J. Biol. Chem.* **255**, 4691–4697.
33. Laurell, C. B. & Eriksson, S. (1963) *Scand. J. Clin. Invest.* **15**, 132–140.
34. Eriksson, S. (1964) *Acta Med. Scand.* **175**, 197–205.