STRUCTURE AND PHOSPHORYLATION OF AN ACIDIC PROTEIN FROM 60S RIBOSOMES AND ITS INVOLVEMENT IN ELONGATION FACTOR-2 DEPENDENT GTP HYDROLYSIS

A.J. van Agthoven, J.A. Maassen and W. Möller

Laboratory for Physiological Chemistry
Sylvius Laboratories
State University of Leiden

Received June 20,1977

SUMMARY: 60S ribosomes from Artemia salina contain two acidic proteins eL12 and eL12(p) of closely related structure and function. The two proteins contain an identical amino acid sequence at the N-terminal region and they are immunologically indistinguishable. The N-terminal amino acid residue is in either case free methionine. Phosphorylation fully explains their slight difference in isoelectric point. No immunological relationship could be detected between eL12/eL12(p) and the 50S protein L7/L12 from Escherichia coli. Treatment of 80S ribosomes from Artemia salina with antibodies against eL12/eL12(p) results in inhibition of elongation factor-2 dependent GTP hydrolysis and polyphenylalanine synthesis. At least one other protein which is structurally related to eL12/eL12(p) is present in the large subunit of Artemia salina ribosomes. This additional protein is slightly larger, judged on the basis of SDS gel electrophoresis.

INTRODUCTION: There is strong evidence that the ribosomal proteins designated L7 and L12 of Escherichia coli are involved in the interaction with the elongation factors EF-Tu and EF-G during the process of protein synthesis (1). The two proteins are identical except that L7 is the N-acetylated form of L12 (2). Together they are present as four copies in the large subunit of the bacterial ribosome (3,4). The general importance of L7/L12 in ribosome function is strengthened by the observation that similar proteins have been found in a number of widely different eukaryotic cells (5,6,7). For instance it has been reported that rat liver ribosomes contain a protein which is antigenetically related to L7/L12 (5) and that in yeast bacterial protein L7/L12 could replace the eukaryotic protein in at least some of the partial reactions of protein synthesis.

Recently this laboratory has focussed on the structure and function of

Abbreviations: EF-G, elongation factor G; EF-Tu, elongation factor Tu; EF-1, elongation factor 1; EF-2, elongation factor 2; eL12, eukaryotic protein, homologous to E.coli L12 (7); eL12(p) phosphorylated form of eL12.

ISSN 0006-291X

two acidic proteins of 60S ribosomes from Artemia salina. The two proteins are structurally related and have been partially characterized (7). Reconstitution experiments indicated that they are involved in EF-1 dependent binding of aminoacyl transfer RNA to ribosome·mRNA complexes (7). Recently Zinker and Warner (8) provided evidence that 60S ribosomes from yeast contain two phosphorylated proteins which judged by their molecular weight and electrophoretic position in two-dimensional polyacrylamide gels may correspond to the ribosomal proteins L7/L12 from E.coli.

In this paper we sought to establish whether phosphorylation of acidic proteins occurs in the embryos of the brine shrimp Artemia salina. Our experiments show that the two proteins which were previously designated as EL7 and EL12 differ by a single phosphate group. In addition we have found that EL7 and EL12 have an identical sequence at least for the first six residues and that in both cases the amino terminal is an unblocked methionine. For this reason we now prefer to call these proteins eL12(p) and eL12 respectively.

We also provide further evidence that eL12/eL12(p) from the large ribosomal subunit of Artemia salina has a function which is similar to that of L7/L12 in bacteria. The eukaryotic acidic protein is involved in elongation factor 2 dependent GTP hydrolysis.

In addition to eL12/eL12(p), we isolated two other 60S acidic proteins which, although immunologically related to eL12/eL12(p), differ from it in other respects.

MATERIALS AND METHODS: 80S ribosomes were prepared and washed as described earlier (7). 80S rather than 60S ribosomes were extracted according to a procedure first described by Hamel and Nakamoto (9) for E.coli ribosomes. The protein extract was next applied to a CMC column to remove any basic ribosomal proteins. The acidic protein fraction was then subjected to DEAE chromatography under conditions which optimize the yield of eL12/eL12(p). Details of the purification will be published elsewhere (10). E.coli L7/L12 was prepared according to Möller et al (11).

Alkaline phosphatase was obtained from Boehringer; aminopeptidase M and Carboxypeptidase C from Röhm and Haas and pronase from Serva.

The presence of phosphate bound to eL12(p) was demonstrated as follows. The acidic protein fraction obtained after CMC chromatography was desalted on a Sephadex G25 column, equilibrated against 0.1% formic acid, and lyophilized. Samples of 100 µg of this acidic protein fraction were dissolved in 50 µl of 1 mM $(NH_4)_2CO_3$ pH 9.0, alkaline phosphatase was added and the mixture incubated at room temperature for 10 minutes, after which the reaction was stopped by freezing. After lyophilization, the samples were analyzed by isoelectric focussing (11).

Inorganic phosphate was determined according to Ames and Dubin (12). The purified proteins eL12 and eL12(p) were desalted on Sephadex G25 in order to remove any free inorganic phosphate present. NaH_2PO_4 was used as a standard in the phosphate determination. For the calculations of the molar ratio of phosphate to protein a molecular weight of 13,000 was assumed for eL12 and eL12(p) (7).

Enzymatic hydrolysis of eL12 or eL12(p) (160 µg) was performed in a solution of 100 µl of 0.1 M Tris. HCl (pH 7.5); 0.05 M KCl, which contained a trace of thymol. The following amounts of enzymes were added: 7 µg of pronase, 7 µg of aminopeptidase M (after 24 hrs), 7 µg of carboxypeptidase C (after 48 hrs). The reaction was terminated after 72 hrs. The temperature was kept at 37°C.

Phosphoserine was detected by electrophoresis on Whatman 3 MM paper dipped in formic acid (2.5%), acetic acid (7.8%) pH 1.9 (13). After drying the paper was sprayed with a ninhydrin solution (0.3% ninhydrin, 3% collidine and 10% acetic acid) and dried for 5 minutes at 120°C.

The N terminal sequence, was established by the dansyl-Edman procedure in the presence of SDS according to Weiner et al (14). Protein was determined by the Lowry procedure (15). Antiserum against eL12(p) or L7/L12 was prepared by injecting on days one and fourteen 1 mg of eL12(p) or L7/L12 in complete Freund's adjuvant intramuscularly in randomly bred rabbits. On day twenty-eight, 0.5 mg of the protein was injected. The rabbits were bled on day fourty-nine. The IgG fraction was purified essentially according to Levy and Sober (16). The percentages of antibodies in the IgG fractions were determined by radioimmune assay using an excess of tritium labeled antigen. The amount of anti eL12(p) was 5.1% and of anti L7/L12 5.6%. Ouchterlony double diffusion tests (17) were performed in 1% agar, 20 mM Borax (pH 8.5) and 0.15 M NaCl. EF-2 from A.salina was prepared from the post-ribosomal supernatant by ammonium sulphate fractionation (30-70%), followed by chromatography on DEAE-cellulose, hydroxylapatite and Sephadex-G100. The EF-2 was free of EF-1 and GTPase activity. A sample of purified EF-1 was kindly donated by Dr. L.I. Slobin. EF-2 dependent GTP hydrolysis was determined in a final volume of 50 µl, containing: 10 mM TRIS-HCl (pH 7.6); 4.5 mM Mg $(OAc)_2$; 90 mM KCl; 0.1 mM EDTA; 6 mM 2-mercaptoethanol; 250 pmoles of [γ-^{32}] GTP (1100 cpm/pmole; Amersham); 0.72 µg of EF-2 and 4 pmoles of 0.5 M KCl-washed 80S ribosomes. Ribosomes were preincubated with antibodies for 10' at 37°. GTP hydrolysis was carried out for 30' at 37°. Subsequently inorganic phosphate was determined according to Kolakowsky et al (18). Polyphenylalanine synthesis was performed in a final volume of 50 µl, containing: 10 mM TRIS-HCl (pH 7.6); 4.5 mM Mg $(OAc)_2$; 90 mM KCl; 6 mM 2-mercaptoethanol; 0.1 mM EDTA; 0.5 mM GTP; 6 µg of polyuridylic acid: 0.5 µg of EF-1; 0.72 µg of EF-2; 30 pmoles of [^{3}H] phenylalanyl-tRNA (1000 cpm/pmole) and 1.5 pmoles of 0.5 M KCl washed 80S ribosomes. Preincubation of ribosomes with antibody was for 10' at 37°C. Synthesis of polyphenylalanine was carried out for 20' at 37°, after which hot TCA insoluble material was determined by filtration through GF/C filters (Whatman).

RESULTS: An indication that eL12(p) is the phosphorylated form of eL12 was obtained by incubating a partial purified preparation of eL12/eL12(p) with increasing amounts of alkaline phosphatase. Subsequent analysis of the digest on isoelectric focussing gels showed a gradual conversion of the band corresponding to the position of eL12(p) into one corresponding to eL12 (fig.1). Since no intermediate forms were observed, a simple explanation would be that removal of a single phosphate group converts eL12(p) into eL12. Inorganic phosphate determination of both proteins supports this hypothesis (table 1). Furthermore phosphoserine was detected after enzymatic hydrolysis of eL12(p); no phosphoserine was found in eL12.

Using antibodies raised against a highly purified preparation of eL12(p)

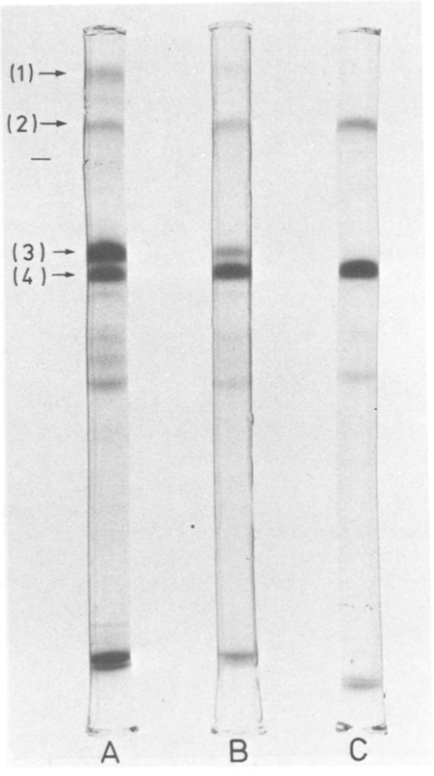


Fig.1 Effect of alkaline phosphatase on the acidic proteins of Artemia salina ribosomes as shown by isoelectric focussing on 5% polyacrylamide gels containing 6M urea and 2% ampholine pH 6-4 (L.K.B. instruments).
A. Control without addition of alkaline phosphatase.
B. Incubation in the presence of 0,016 µg alkaline phosphatase.
C. Incubation in the presence of 0,125 µg alkaline phosphatase.
arrow 1, topband 1; arrow 2, topband 2; arrow 3, eL12(p); arrow 4, E112.

Table 1

Inorganic phosphate determination of two different preparations eL12 and EL12(p).

	Mol phosphate/mol protein	
	prep.1	prep.2
eL12	0,08	0,02
eL12(p)	1,10	1,02

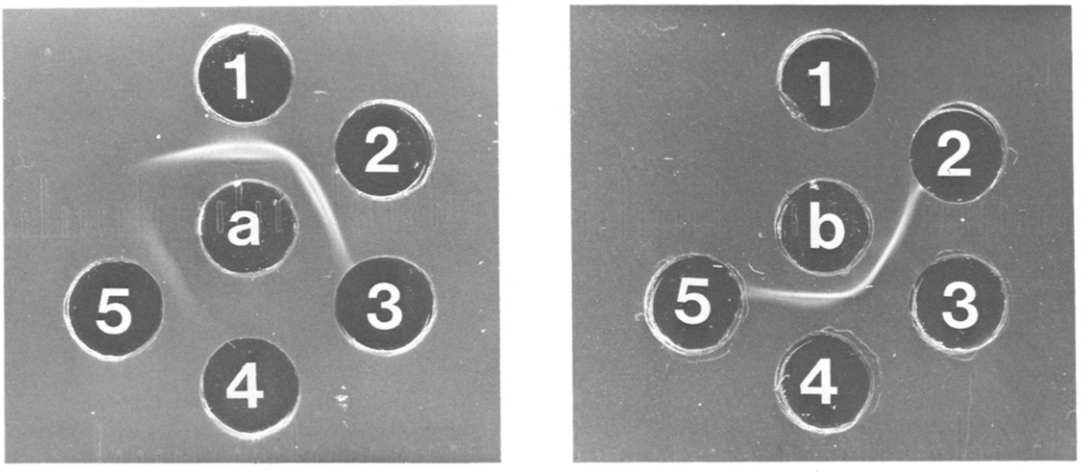


Fig.2 Ouchterlony immunodiffusion: reaction of antisera to eL12(p) and to L7 with eL12, eL12(p), L7, L12 and a 1:1 mixture of topband protein 1 and 2. The center wells contained IgG: in a, anti eL12(p) (625 µg); in b, anti L7 (500 µg). The peripheral wells contained in 1, eL12 (5 µg); in 2, eL12(p) (5 µg); in 3, L7 (5 µg); in 4, L12 (5 µg); and in 5, 2,5 µg of each topband protein 1 and 2.

it was found that anti eL12(p) precipitated eL12(p) as efficiently as eL12. (fig.2).

When the amino terminal region of eL12 was compared to that of eL12(p) it became evident that the sequence of the first six amino acids - met,arg, tyr, val,ala, ala - from the N-terminal end was identical in both proteins. Free methionine was found at the N-terminal position.

So far, we have not been able to observe any immunological cross-reaction between Artemia salina eL12 and E.coli L7/L12. Neither the reaction between anti Artemia eL12(p) and E.coli L7/L12 nor the reaction between anti E.coli L7 and Artemia eL12(p) met with any success. For this purpose we used Ouchterlony double-diffusion assays (fig.2).

The Artemia eL12(p) antibodies were also tested against the two other acidic proteins which were even more acidic than eL12/eL12(p) and are positioned near the top (anodic side) of an isoelectric focussing gel (fig.1). Interestingly, anti-eL12(p) shows a definite cross-reaction with the proteins of this very acidic protein fraction. However, the reaction was less strong than in the case of eL12/eL12(p) (fig.2). A positive cross-reaction was also obtained when each of these topband proteins 1 and 2 were tested separately against anti eL12(p) (results not shown). It was also noteworthy that incubation of a partially purified preparation of acidic

Table 2

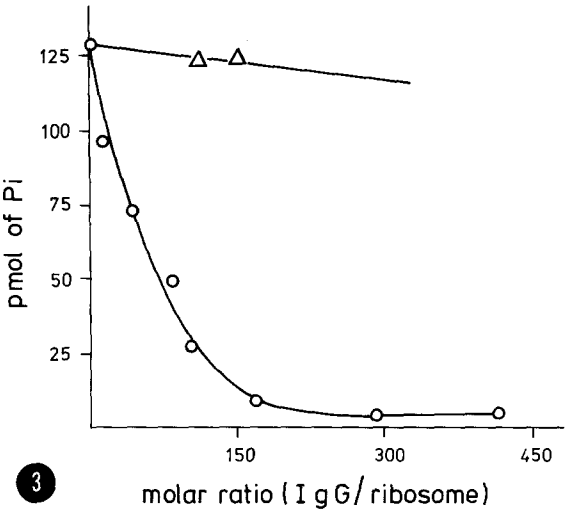
Amino acid composition of eL12/eL12(p) and of a 1:1 mixture of the more acidic topband proteins 1 and 2 from 60S subunits of Artemia salina. Compositions are given in mol. percent. (Significant differences in contents are underlined).

amino acid	eL12	topband protein
Lys	9.1	8.4
His	0.0	0.0
Arg	0.8	0.7
Asx	8.7	10.2
Thr	2.8	5.5
Ser	5.5	5.3
Glx	17.3	14.9
Pro	3.9	3.2
Gly	10.6	10.7
Ala	20.5	16.9
Val	2.6	5.7
Met	2.7	2.5
Ile	3.6	3.8
Leu	9.2	9.1
Tyr	1.0	0.9
Phe	1.4	2.3
Lys	0.0	0.0

proteins with alkaline phosphatase results in the disappearance of topband protein 1 from the isoelectric focussing gel (fig.1). It is not unlikely that this protein which is related to eL12 is also phosphorylated.
A 1:1 mixture of topband proteins 1 and 2 gave on SDS gel electrophoresis one single band. This band moved just behind that of eL12/eL12(p), thereby suggesting a molecular weight in excess of that of eL12/eL12(p).

In addition, the tryptic peptide maps of these topband proteins were similar to each other and clearly related to those of eL12/eL12(p) (results not shown). The amino acid composition of each of the topband proteins was also similar to each other but indicated significant differences with eL12 (table 2); moreover free methionine could not be detected at the N-terminal as in eL12.

The relative amount of topband protein 1/2 to protein eL12/eL12(p) varied, although eL12/eL12(p) was always the predominant protein. To see whether the topband proteins were immunologically related to E.coli L7/L12, they were tested against anti E.coli L7/L12 but the results were again negative(fig.2). The 60S P1-split proteins from Artemia salina, prepared according to ref.9 also gave a negative cross-reaction. Controls of different preparations of



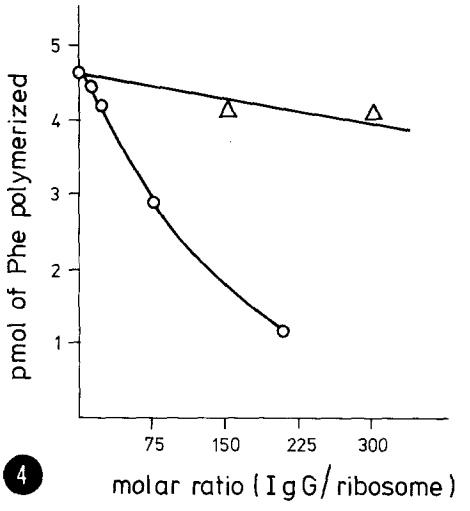


Fig.3 Inhibition of EF-2 dependent GTPase by anti eL12(p).

Incubation of 4 pmoles of 80S ribosomes with IgG fraction against A.salina eL12(p) (-0-0-) or E.coli L7/L12 (-Δ -Δ -). The IgG fractions contained 5.1% anti eL12(p) or 5.6% anti L7/L12 (see Materials and Methods).
Incubation of ribosomes with IgG from non-immunized rabbits had no significant effect.

Fig.4 Effect of anti eL12(p) on polyphenylalanine synthesis. Incubation of 1.5 pmoles of 80S ribosomes with anti eL12(p) immunoglobuline (-0-0-) or non-immune IgG (-Δ-Δ-).

E.coli L7/L12 and Artemia eL12/eL12(p) combined with their homologous antibodies gave positive cross-reactions in all cases.

Fig.3 shows the inhibitory effect of preincubation of 80S ribosomes with IgG against eL12(p) on the EF-2 dependent GTP hydrolysis reaction. From this figure it can be calculated that an eightfold molar excess of anti eL12(p) to ribosomes gives almost complete inhibition of GTP hydrolysis. Incubation of ribosomes with IgG from non-immunized rabbits had no significant effect (results not shown). In addition anti E.coli L7/L12 also did not inhibit GTP-ase activity (fig.3). If anti eL12(p) is preincubated with increasing amounts of antigen the inhibitory effect of the antibody disappears (not shown). This indicates that the inhibitory effect is due to a specific binding of the antibody to the acidic protein on the ribosome.

Anti eL12(p) also inhibited polyphenylalanine synthesis (fig.4). Furthermore, as was the case for GTPase activity, preincubation of the antibody with the eL12(p) decreased the inhibitory effect (not shown).

DISCUSSION: The data presented confirm an earlier report that 60S ribosomes from Artemia salina contain two acidic proteins of related structure (7). They establish that in contrast to E.coli L7/L12 (2) the N-terminal residue of Artemia eL12/eL12(p) is unblocked methionine and not partially acetylated serine. Phosphorylation rather than N-terminal acetylation (2) seems responsible for the difference in isoelectric points of Artemia eL12 and eL12(p). Previous studies (19) dealing with the effects of a protein kinase on the E.coli 50S ribosome demonstrated that in vitro only L7 and L12 among the 34 different 50S proteins were phosphorylated in the presence of [γ^{32}P]-GTP. Perhaps phosphorylation of eukaryotic eL12 takes place for reasons which reflect its special exposure on the ribosome (20).

Although a definite precipitation line was found between anti E.coli L7/L12 and rat liver L40/L41 - the homologous protein to E.coli L7/L12 (5,21)-, no cross-reaction could be detected in the case of anti E.coli L7/L12 and Artemia eL12/eL12(p). Similarly no immunological crossreactivity was observed between anti E.coli L7/L12 and L20, - the protein homologous to E.coli L7/L12 in Halobacterium curtirubrum - notwithstanding its sequence homology with E.coli L7/L12 (22). Since there are no a priori reasons why L40 and L41 - the protein of rat liver homologous to L7/L12 - is immunologically more related to the acidic protein of E.coli than that of H.cutirubrum or Artemia, this result is somewhat unexpected. Sequence analysis of the Artemia eL12/eL12(p) protein should unambiguously determine the actual degree of homology with E.coli L7/L12 and even predict (23) the extent of expected immunological resemblance.

Another interesting aspect of our study concerns the occurrence of a group of two proteins which show structural characteristics similar to eL12. Although their characterization is much less complete, their lower mobility on SDS gels suggests a possible precursor relationship with eL12.

Since completion of our work it has been reported that proteins which may be related to E.coli L7/L12 also occur in Krebs II ascites cells and that in this case the protein is also phosphorylated (24). It has been firmly established that L7/L12 is involved in the process of elongation factor dependent GTP hydrolysis in E.coli(1,25). A similar function has been suggested for the acidic ribosomal proteins from eukaryotes (5,7). The results in this paper provide further support for this suggestion.

Since, as expected, both eL12 and eL12(p) fully crossreact with anti eL12(p) only reconstitution experiments will establish whether phosphorylation of eL12 is involved in the GTP dependent reactions of the elongation step(s) in eukaryotic protein biosynthesis.

During the preparation of this manuscript, an article by Howard et al(26)

appeared in which it is shown that antibodies against E.coli L7/L12 inhibit the elongation factors dependent processes in chicken liver ribosomes. Although their conclusions concerning the involvement of the acidic proteins in the elongation step of eukaryotes are in agreement with our findings, to date we have been unable to find any effect of anti E.coli on the EF-2 dependent GTPase reaction in Artemia salina.

ACKNOWLEDGEMENTS: We wish to thank Dr. L.I. Slobin for advice; Mr. J. Kriek for preparing the acidic proteins and Miss J.D.J. Steeneveld for excellent technical assistance.

REFERENCES

(1) Möller, W. (1974) in "Ribosomes", eds. Nomura, M., Tissières, A. and Lengyel, P. (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.) pp. 711-731.

(2) Terhorst, C., Möller, W., Laursen, R., Wittmann-Liebold, B.(1973) Eur.J.Biochem. 34, 138-152.

(3) Subramanian, A.R. (1975) J.Mol.Biol. 95, 1-8.

(4) Hardy, S.J.S. (1975) Mol.Gen.Genet. 140, 253-274.

(5) Stöffler, G., Wool, I.G., Lin, A. and Rak, K.H. (1974) Proc.Nat.Acad.Sci.U.S.A. 71, 4723-4726.

(6) Richter, D. and Möller, W. in "Lipmann Symposium" (1974) (D. Richter, ed.) p. 524-533. de Gruyter, Berlin.

(7) Möller, W., Slobin, L.I., Amons, R and Richter, D. (1975) Proc.Nat.Acad.Sci. U.S.A. 72, 4744-4748.

(8) Zinker, S. and Warner, J.R. (1976) J.Biol. Chem. 251, 1799-1907.

(9) Hamel, E., Koka, M. and Nakamoto, T. (1972) J.Biol.Chem. 247, 805-814.

(10) van Agthoven, A.J. and Möller, W. in preparation.

(11) Möller, W., Groene, A., Terhorst, C.P., Amons, R. (1972) Eur.J.Biochem. (25, 5-12.

(12) Ames, B.N., Dubin, D.T. (1960) J.Biol.Chem., 235, 769-775.

(13) Langan, T.A. (1968)
Regulatory Mechanisms for Protein Synthesis in Mammalian Cells.
New York, Acad.Press. p.10

(14) Weiner, A.M., Platt, T. and Weber, K. (1972)
J.Biol.Chem. 247, 3242-3251.

(15) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951)
J. Biol.Chem., 193, 265-275.

(16) Levy, H.B. and Sober, H.A. (1960)
Proc.Soc.Exp.Biol.Med. 103, 250-252

(17) Ouchterlony, O. (1968)
Handbook of Immunodiffusion and Immunoelectrophoresis (Ann Arbor Science Publishers, Ann Arbor, Mich.)

(18) Kolakowsky, D., Dewey, K.F., Hershey, J.W.B. and Thach. R.E. (1968)
Proc.Nat.Acad.Sci. U.S.A., 61, 1066-1070.

(19) Issinger, O.G. and Traut, R.R. (1974)
Bioch. Biophys. Res. Comm. 59, 829-836.

(20) Lake, J.A. (1976)
J.Mol.Biol. 105, 131-159.

(21) Sherton, C.C. and Wool, I.G. (1974)
J.Biol. Chem. 249, 2258-2267.

(22) Oda, G., Strøm, A.R., Visentin, L.P. and Yaguchi, M. (1974)
FEBS Letters, 43, 127.

(23) Prager, E.M. and Wilson, A.C. (1971)
J.Biol.Chem. 246, 7010-7017.

(24) Leader, D.P. and Coia, A.A. (1977)
Biochem.J. 162, 199-200.

(25) Highland, J.H., Ochsner, E., Gordon, J., Bodley, J., Hasenbank,R. and Stöffler, G. (1974)
Proc.Nat.Acad.Sci. U.S.A., 627-630.

(26) Howard, G.A., Smith, R.L. and Gordon, J. (1976)
J.Mol.Biol. 106, 623-637.