AMINO ACID ACYLASES: PROPOSED RELATION TO METABOLIC RECYCLING OF METHIONINE DURING PROTEIN BIOSYNTHESIS

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Received April 11, 1967

Evidence from several laboratories indicates that Escherichia coli uses N-formylmethionine-tRNA during initiation of polypeptide chain biosynthesis (Capecchi, 1966; Clark and Marcker, 1966a; Eisenstadt and Leyngyel, 1966; Leder and Bursztyn, 1966; Nakomoto and Kolakofsky, 1966; Noll, 1966; Revel and Gros, 1966; Salas et al., 1967; Thach et al., 1966; Webster et al., 1966). The N-formylmethionine is produced by formylation of a methionyl-$tRNA_F$ intermediate differing from a second methionyl-$tRNA_M$ which is not formylated (Clark and Marcker, 1966b). The presence of the N-formylmethionine at the N-terminus of the final product has only been reported in the instance of a cell-free system synthesizing a bacteriophage coat protein (Clark and Marcker, 1966b). These findings suggest that at termination of polypeptide chain biosynthesis the N-formylmethionine is cleaved from the N-terminal position. An accumulation of N-formylmethionine could result in depletion of cellular methionine; the hydrolysis of N-formylmethionine and subsequent return of the free methionine to the metabolic pool would be expected, but has not yet been reported. We are presenting preliminary evidence indicating that the N-acylamino-acid amidohydrolases, such as the well-studied renal acylase I (E.C. 3.5.1.14), could serve this function. A similar enzyme isolated and purified 100-fold from the combined cotyledon and embryo of seeds of the Palo Verde tree, or Jerusalem-Thorn (Parkinsonia aculeata L.) was

found to hydrolyze specifically N-acylamino acids, but possessed the highest activity when N-formylmethionine was the substrate. Determination of kinetic constants using various substrates showed that the enzyme had the highest V_{max}/K_m (physiological efficiency) for N-formylmethionine. This amino acid acylase appears to be similar to other acylases isolated from hog kidney, yeast, molds, and various seeds (Cheng-Wu and Orekhovich, 1957; Greenstein and Winitz, 1961; Meister, 1965; Osaki and Wetter, 1960). Despite the ubiquity of this type enzyme, no physiological function has heretofore been attributed to it.

Materials and Methods:

The enzyme source was seeds harvested in suburban Phoenix, Arizona in the summer of 1966. The seeds were soaked in water overnight to soften the coat. The embryo and cotyledon fraction was removed from the seed coat and enveloping gelatinous layer, then homogenized in a Waring blendor with chilled acetone (-15°C). The dried acetone powder was stable up to six months when kept frozen at -15°C. The powder was extracted with phosphate buffer (0.1M, pH 7.5) containing Co^{2+} (4×10^{-4}M), and the extract was centrifuged at 7,000 rpm for 15 minutes. This and all subsequent operations were done at 0-to-4°C unless otherwise specified. The crude extract was heated in a water bath at 45°C for 10 minutes and immediately cooled in ice-water. Inactive protein was removed by centrifugation and then $(NH_4)_2SO_4$ precipitation was carried out twice. The fraction precipitating between 35-to-50% saturation was dissolved in minimum amount of water and dialyzed overnight against tap water. Acetone precipitation of the active fraction was carried out twice. The fraction precipitating between 20-to-25% by volume (based on the volume of the dialyzed solution) was dissolved in minimum amount of buffer and used throughout the experiment. The purification obtained was about 100-fold. Frozen aliquots of enzyme retained activity indefinitely.

Results and Discussion:

As shown in Table I, the rate of hydrolysis for N-formylmethionine is

Table I

Substrate Reactivity With Palo Verde Acylase

Amino Acid	Hydrolysis-Rate for N-acetyl-	Hydrolysis-Rate for N-formyl-	Ratio of N-formyl- to N-acetyl-	N-formylmethionine Rate Ratio to N-formyl-	N-formylmethionine Rate Ratio to N-acetyl
L-methionine	550	1,750	3.18	1.00	3.18
L-alanine	517	390	0.76	4.50	3.39
L-valine	943	127	0.14	13.80	1.86
L-leucine	875	738	0.85	2.37	2.00
L-phenylalanine	139	100	0.72	17.50	12.60
L-tyrosine	73	24	0.33	73.00	24.00
glycine	276	92	0.33	19.03	6.35
L-glutamic acid	187				9.36
L-histidine	165				10.61
L-tryptophan	2.5				

Reaction tube contained substrate (0.05 M), Co^{2+} (4×10^{-4} M), and enzyme (145 µg), all in 1.50 ml phosphate buffer (pH 7.2, 0.1 M). Incubation was for 30 min at 37°C. Aliquots (0.1 ml) were assayed for increase in A_{570} by the standard ninhydrin method of Moore and Stein (1948). Rate values are micromoles substrate hydrolyzed/hour/mg protein nitrogen.

far greater than all the other derivatives. We also observed that a commercial preparation of renal acylase I hydrolyzed N-formylmethionine 1.6 times faster than it did N-acetylmethionine. When a comparison was made of the rate of hydrolysis of the formyl and acetyl derivatives of methionine and of alanine at pH 7.2 and pH 9.0 (Table II), the formyl derivatives were hydrolyzed more efficiently under the physiological conditions. Kinetic constants were determined and the results expressed as the physiological efficiency (V_{max}/K_m) of the enzyme with several substrates (Table III). It was shown that N-formylmethionine had the most favorable Michaelis constant. Extrapolation of a Lineweaver-Burke plot gives an apparently

Table II

Kinetic Constants of Palo Verde Acylase With Four Substrates

Substrate	K_m	V_{max}	V_{max}/K_m
N- acetyl-L-methionine	2.50×10^{-2}	*2.50×10^{3}	1.00×10^{5}
N- acetyl-L-alanine	4.45×10^{-3}	6.67×10^{2}	1.50×10^{5}
N- formyl-L-methionine	2.92×10^{-3}	1.17×10^{3}	3.98×10^{5}
N- formyl-L-alanine	3.23×10^{-2}	5.56×10^{2}	1.74×10^{4}

*N- Acetyl-L-methionine exhibits substrate inhibition at .005 molar and higher concentrations; the tabulated V_{max} value is extrapolated from a Lineweaver-Burke plot. Reaction tubes contained an appropriate concentration of substrate and 320 µg enzyme and other components as mentioned in Table I. Both the enzyme and substrate were preincubated at 37°C, mixed, and then incubated for 15 min. Assay procedure and expression of rate values as described in Table I.

Table III

Hydrolysis Rates at Two pH Values

Substrate	Hydrolysis Rate	Relative Rate
*pH 7.2		
N-acetyl-DL-methionine	380	1.00
N-acetyl-DL-alanine	505	1.33
N-formyl-DL-methionine	1,580	4.15
N-formyl-DL-alanine	487	1.28
pH 9.0		
N-acetyl-DL-methionine	420	1.11
N-acetyl-DL-alanine	825	2.18
N-formyl-DL-methionine	519	1.37
N-formyl-DL-alanine	293	0.77

*In these experiments all substrates were DL, so both the hydrolysis rates and the relative rates are not exactly comparable with those in Table I. Reaction tubes contained a total volume of 3.0 ml with 0.05 molar L-substrate; preincubation and incubation for 30 min were as described in Tables I and II except that 192 µg enzyme was used and the pH 9.0 experiments were in 0.1 molar pyrophosphate buffer.

higher V_{max} for N-acetylmethionine, but at high substrate concentrations it exhibits an inhibitory effect on the enzyme. The physiological efficiency

values indicate N-formylmethionine to be the more favorable substrate. A mold acylase has been reported (Borkar, 1957) which hydrolyzes N-formyl-amino acids faster than other substrates, and an E. coli preparation hydrolyzed N-formylglycine (Imaizumi, 1938). Vogel and Bonner (1956) reported that the E. coli α-N-acetylornithinase also hydrolyzed N-acetylmethionine, but the Palo Verde enzyme must be different since it does not use α-N-acetylornithine as a substrate. Both N-acetyl-L-leucine and N-acetyl-L-valine are better substrates than N-acetyl-L-methionine (see Table I), yet their corresponding N-formyl-derivatives are less active than both the N-acetyl- and N-formylmethionines; the enzyme must be showing some degree of selectivity greater than what might be expected based upon ease of chemical hydrolysis of N-formyl- vs. N-acetyl-derivatives. The kinetic data presented in Tables II and III certainly argue for N-formylmethionine as the best substrate under normal conditions. Although no definite evidence has yet been reported to implicate N-formylmethionine in peptide chain initiation in organisms other than E. coli, we are led to conclude that the general function of acylases is the hydrolysis of N-formylmethionine to recycle the methionine back into the metabolic pool during protein synthesis.

Acknowledgment: The authors gratefully acknowledge the financial assistance furnished to J. N. A. by the Arizona Academy of Science in order that seeds could be acquired.

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