ENZYMATIC SYNTHESIS OF DIADENOSINE TETRAPHOSPHATE AND DIADENOSINE TRIPHOSPHATE WITH A PURIFIED LYSYL-sRNA SYNTHETASE

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In a study of the lysine amino acid activation reaction in protein synthesis, we have employed optical rotatory dispersion (ORD) to look for physical evidence of a possible change in conformation of l-lysine:sRNA ligase (AMP), (EC 6.1.1.6). When lysine activation was carried out in a thermostated cell in the Cary 60 spectropolarimeter, a Cotton effect developed during a 30 minute incubation at 37°C. The appearance of this effect was dependent on the presence of the lysyl-sRNA synthetase, lysine, ATP, and Mg^{++}, and the effect was increased by raising the ATP concentration and by addition of pyrophosphatase. Pressure dialysis of the reaction mixture following incubation revealed that a dialyzable material was responsible for this ORD effect (Fig. 1).

When the dialyzable material was lyophilized, taken up in water, and subjected to thin-layer chromatography (TLC) on poly(ethyleneimine)-cellulose (PEI-cellulose) (Randerath and Randerath, 1965, 1966), an unidentified new compound, quenching short-wave ultraviolet light, was observed. It appeared that ATP was being converted into this compound during the enzymatic reaction. A few μmoles of the new nucleotide were isolated by preparative TLC for subsequent analysis. The compound has been identified as P^1,P^4-di(adenosine-5')tetraphos-

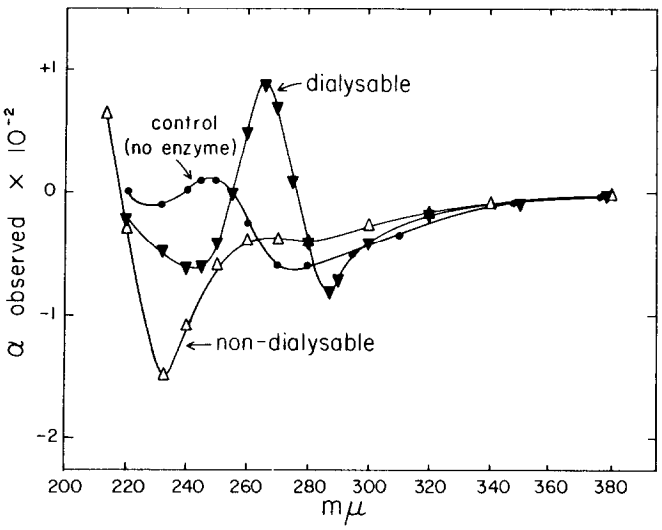


Fig. 1. ORD curves obtained after incubation of lysyl-sRNA synthetase with ATP and lysine. The incubation was carried out in a total volume of 2 ml at 37°C for 2 hours. The reaction mixture contained 0.05 M Tris-HCl at pH 7.4, 0.01 M $MgCl_2$, 0.0015 M ATP, 0.0015 M L-lysine, 10 μg of pyrophosphatase (Worthington)/ml, and 220 μg purified lysyl-sRNA synthetase/ml. The lysyl-sRNA synthetase was prepared by the method of Stern and Mehler (1965) and 1 μg was able to esterify about 1.5 mμmoles of lysine to sRNA in 10 min at 25°C. Following incubation the solution was chilled and 0.02 M EDTA was added, since the Cotton effect has the greatest amplitude at 5°C and in the presence of EDTA. The incubation mixture was subjected to pressure dialysis to separate the protein from smaller molecules, and the concentrated protein inside the bag was diluted to the original volume with 0.05 M Tris, 0.01 M $MgCl_2$. ORD curves were obtained at 5°C on the dialysate, the non-dialyzable fraction, and the original incubation mixture minus the protein. The figure indicates that the dialyzable material exhibits a large Cotton effect.

phate (APPPPA or AP_4A) (cf. Randerath et al., following paper). This compound possesses the ORD properties - i.e., the Cotton effect - previously observed. A smaller amount of P^1,P^3-di(adenosine-5')triphosphate (APPPA or AP_3A) has also been found.

The compound AP_4A is formed in small amounts from ATP in the presence of a purified (but not homogeneous) preparation of E. coli lysyl-sRNA synthetase (Stern and Mehler, 1965) even without the addition of exogenous l-lysine (cf. Table 1). It is formed maximally when

TABLE 1. NUCLEOTIDES FORMED DURING AN INCUBATION OF αP^{32}-ATP WITH LYSYL-sRNA LIGASE

							E. coli sRNA Added				
	Hours	ATP	APPPPA	ADP	APPPA	AMP	ATP	APPPPA	ADP	APPPA	AMP
Control											
No amino acid	0	4,424	-	74	-	-	4,098	-	67	-	-
10 μg enzyme	1.5	4,049	166	134	12	15	4,032	23	167	8	9
	3.0	3,577	215	209	11	15	3,943	34	207	8	8
	13.0	3,606	772	454	30	39	3,458	32	497	11	37
0.0015 M L-lysine											
10 μg enzyme	0	4,405	-	64	-	-	3,787	-	47	-	-
	1.5	3,205	738	200	53	85	1,727	2,065	181	57	92
	3.0	2,634	1,454	365	57	186	748	2,765	267	115	170
	13.0	255	2,970	698	481	531	193	2,440	370	683	693
0.0015 M L-arginine											
10 μg enzyme	0	4,566	-	65	-	-	4,695	-	57	-	-
	1.5	3,484	557	199	38	75	3,401	253	282	41	235
	3.0	2,635	832	351	40	301	2,732	316	463	40	441
	13.0	1,010	1,523	838	115	823	2,117	370	1,176	40	781
0.0015 M L-lysine											
5 μg enzyme	0	4,587	-	57	-	-	4,901	-	63	-	-
	1.5	4,000	376	163	21	49	2,907	1,288	136	49	72
	3.0	3,466	717	231	24	91	1,785	2,103	215	60	143
	13.0	1,279	2,534	533	171	230	292	3,337	392	291	482
0.0015M L-lysine											
20 μg enzyme	0	4,219	-	68	-	-	4,608	-	68	-	-
	1.5	2,778	1,210	322	106	168	885	2,948	200	128	143
	3.0	1,382	2,140	468	151	338	258	3,281	272	260	288
	13.0	138	1,689	873	950	798	163	1,051	497	1,285	1,051

Incubations were carried out at 37°C in a total volume of 0.05 ml. Each tube contained 0.04 M Tris-HCl at pH 7.8, 0.008 M $MgCl_2$, 0.002 M ATP (αP^{32}-ATP (Schwarz) containing 200 cpm/mμmole) and 10 μg of enzyme protein, except where noted. Mixed, stripped *E. coli* sRNA was added in a final concentration of 2 mg/ml where indicated. Aliquots of 10 μl were plated at various time intervals on thin-layer PEI-cellulose sheets, 20 x 20 cm in size. The chromatograms were developed with 1 M LiCl. Nucleotides, identified by their absorbance in ultraviolet light, were cut from the plates and counted, using an end-window gas-flow counter with a background of 2-3 cpm. The results are expressed as total counts in each spot isolated from 10 μl of incubation mixture.

a comparatively small amount of E. coli sRNA (unfractionated) is added to an incubation mixture containing in addition l-lysine, Tris buffer, pH 7.8, $MgCl_2$, and ATP. Under such conditions, no spot indicative of AP_4A is seen in the absence of lysine. Thus, in the presence of sRNA, the formation of AP_4A is strictly dependent on addition of l-lysine to the incubation mixture.

When AP_4A-C^{14}, previously formed enzymatically and isolated by preparative TLC, is incubated with lysine and lysyl-sRNA synthetase without added ATP, ATP-C^{14} is formed in small amount. Thus the enzymatic reaction leading to synthesis of AP_4A is reversible, although the equilibrium position is far toward formation of AP_4A under these circumstances (Table 2). The ATP formed in the reaction is promptly used, being converted via the activation reaction largely to AMP.

When AP_4A is used in place of ATP in a system containing lysyl-sRNA synthetase, C^{14}-lysine, and sRNA, it is able to serve as an energy source for the activation-esterification reactions resulting in lysyl-sRNA (unpublished data). It appears to do so by the mechanism shown in the following paper (Randerath et al., Fig. 4).

As suggested by the kinetic studies of Loftfield and Eigner (1965), the addition of sRNA to the activating enzyme system stimulates both the forward and reverse reactions (cf. Tables 1 and 2).

The reaction in which AP_4A is formed appears to be similar to the reversal of the first step in amino acid activation, in which pyrophosphate reacts with the enzyme-bound aminoacyl-AMP moiety, with the re-formation of ATP and free amino acid. The synthesis of AP_4A is pictured schematically in Fig. 2. A more detailed proposed mechanism for formation of AP_4A and AP_3A is depicted in Fig. 3. Derivatives terminating in a pyrophosphate moiety react with the enzyme-bound aminoacyl AMP, with the formation of nucleotides containing both the AMP and the original pyrophosphate derivative, and with elimination of lysine.

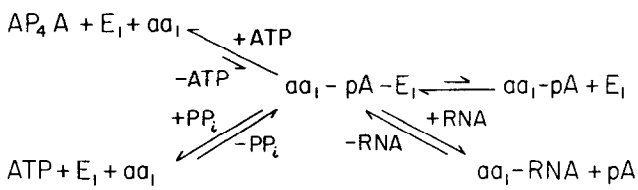
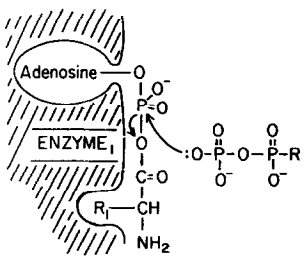


Fig. 2



R	Pyrophosphate derivative	Compound formed
OH	PP_i	ATP
adenosine	ADP	AP_3A
AMP	ATP	AP_4A

Fig. 3

The small amount of AP_3A which is synthesized appears after traces of ADP have been formed during the incubation by a mechanism as yet unclear, possibly due to a phosphatase contaminant.

Whether the formation of AP_4A and related compounds is a property of the lysyl-sRNA synthetase alone, or is shared by other aminoacyl-sRNA synthetases is at the present moment unknown. It can be mentioned that we have not observed synthesis of AP_4A in a similar system containing semi-purified arginyl-sRNA synthetase.

The possibility remains that a contaminating enzyme entirely separate from lysyl-sRNA synthetase may be responsible for formation of AP_4A and related compounds. It is nevertheless tempting to consider that these compounds (cf. Randerath et al., following communication for a description of other compounds of similar structure, such as AP_4dG, which may be formed in this reaction) may serve as energy storage mechanisms, located at the gateway to protein synthesis, insuring that the

energy of amino acid activation may not be lost when the later steps in protein synthesis slow down for other reasons. In a more specific sense, they may be regarded as stable forms of energy primers, convertible to the nucleoside triphosphates by way of the first reaction in protein synthesis. ATP and other ribonucleoside or deoxyribonucleoside triphosphates, having been formed in this way, now become available to set in motion other synthetic processes, such as DNA and RNA synthesis, in the metabolic reawakening of dormant cells.

TABLE 2

Nucleotides Formed During an Incubation of AP_4A-C^{14} with Lysyl-sRNA Synthetase

		Time	APPPPA	ATP	ADP	AMP	APPPA
I.	Control (APPPPA, enzyme, buffer)	7 min	1622	2	11	0	1
		2.0 hr	1757	0	23	2	0
		4.5 hr	1169	1	18	3	0
II.	I + lysine	7 min	1678	2	2	0	0
		2.0 hr	1683	2	34	17	2
		4.5 hr	1630	2	21	73	3
III.	I + sRNA	7 min	1803	3	5	0	2
		2.0 hr	1672	3	12	7	0
		4.5 hr	1775	8	7	13	1
IV.	I + lysine + sRNA	7 min	1706	28	7	24	2
		2.0 hr	1344	43	11	85	1
		4.5 hr	1257	51	19	312	91
V.	IV + 0.00015 M ATP	7 min	1676	17	2	0	0
		2.0 hr	1701	65	6	9	0
		4.5 hr	1608	43	20	129	70
VI.	IV + 0.0015 M ATP	7 min	1754	14	9	0	0
		2.0 hr	1620	52	32	0	5
		4.5 hr	1625	73	26	13	12

Preparative amounts of C^{14}-labeled AP_4A were prepared by incubation of a total volume of 1.0 ml for 4 hours at 37°C. The tube contained 0.04 M Tris-HCl, pH 7.8, 0.008 M $MgCl_2$, 0.0015 M l-lysine, 0.003 M ATP-8-C^{14} (Schwarz), and 2 mg *E. coli* sRNA, stripped and unfractionated. The mixture was applied to one 20 cm square PEI-cellulose sheet. This was soaked in methanol for 10 minutes, air dried and the chromatogram was run in 1 M LiCl. The compound was eluted with 3 N ammonia for 10 minutes at 0°C, lyophilized and taken up in a small volume of water (see Randerath *et al.*, following paper). This compound was free of ATP.

The experiment was carried out as described in Table 1 except that the synthetase concentration was 200 μg protein/ml, and 0.000142 M AP_4A-C^{14} at 1200 cpm/mμmole was added. 0.0015 M l-lysine and E. coli sRNA at 2 mg/ml were added where indicated. There was no ATP added except where shown in the table. The PEI-cellulose containing the compounds was scraped from the plastic sheet, wet with 2 M LiCl, and suspended in 10 ml of scintillation fluid (Bucher, N. L. R., and Swaffield, M. N., personal communication). The samples were counted in a Nuclear-Chicago liquid scintillation counter having a background of 40. Each value represents the total cpm found in the nucleotide from 10 μl of the 50 μl incubation mixture, and includes the variability of the total procedure ($\sigma_{rel} = \pm 4\%$).

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