Acta Chemica Scandinavica B 31 (1977) 95—101

Properties of the Sulfhydryl Groups of Three Amino Acid:Transfer RNA Ligases

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Some properties of the sulfhydryl groups of three amino acid:tRNA ligases have been investigated.** For the valine ligase from yeast SH-groups were found to be essential both for valine activation and aminoacylation of $tRNA^{Val}$. The lysine ligase from yeast required SH-groups for the amino acid activation reaction whereas the transfer of [^{14}C]-lysine from the lysyladenylate–enzyme complex to $tRNA^{Lys}$ was not inhibited by 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2). The lysine ligase from *E. coli* was not sensitive to Nbs_2 neither with regard to lysine activation nor tRNA aminoacylation.

The interactions between amino acid:tRNA ligases and their substrate molecules can be fully understood only in the light of structural information from single crystal analyses now in progress in several laboratories.[1–5] Nevertheless it is useful to characterize these enzymes with respect to their properties in solution and the effect of substrate molecules on these properties. We have previously reported the purification of three amino acid ligases [6–8] as well as some of their molecular and crystallographic parameters.[2,9,10] In this paper we have attempted to study the properties of the sulfhydryl groups of the enzymes and the influence of substrates on enzyme conformation in solution as judged by such properties as accessibility of sulfhydryl groups for SH-reagents, sedimentation velocities and circular dichroism.

MATERIALS AND METHODS

Materials. The valine and lysine:tRNA ligases were prepared using previously described procedures,[6–8] which had been modified to allow the processing of 5 kg of yeast or 3 kg of *E. coli*. Highly purified $tRNA^{Lys}$ and $tRNA^{Val}$ were prepared from crude tRNA from *E. coli* (General Biochemicals) and brewer's yeast (Boehringer-Mannheim) by chromatography of the *N*-phenoxyacetylated aminoacyl-tRNA's on benzoylated DEAE-cellulose according to Gillam *et al.*[11,12] as described in Ref. 2.

Inorganic [^{32}P]pyrophosphate and ^{14}C-labelled amino acids were purchased from the Radiochemical Centre, Amersham, England. 5,5′-Dithiobis(2-nitrobenzoic acid), (Nbs_2), was obtained from Sigma Chemical Company. Guanidine hydrochloride, sequanal grade, was from Pierce Chemical Company. Other chemicals used were of the highest grade available.

Protein determinations. The protein concentrations were determined spectrophotometrically at 280 nm in 0.05 M potassium phosphate buffer, pH 7.0, using the following molar absorption coefficients for the enzymes: lysine:tRNA ligase (yeast) 92 500 M^{-1} cm^{-1}, lysine:tRNA ligase *(E. coli)* 66 600 M^{-1} cm^{-1} and valine:tRNA ligase (yeast) 171 000 M^{-1} cm^{-1}. All calculations in this paper are based on the molecular weights of the enzymes given in Ref. 2.

Reaction of sulfhydryl groups with Nbs_2. Prior to reaction with Nbs_2 the enzymes were incubated with 5 mM dithiothreitol. After 60 min at room temperature the reducing agent was removed by chromatography on Sephadex G-25 equilibrated with 0.5 M potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA and saturated with N_2. This treatment did not significantly change the specific activity of the enzymes. The reaction of the enzymes

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** *Abbreviations:* Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); $tRNA^{Val}$, transfer ribonucleic acid specific for valine; $tRNA^{Lys}$, transfer ribonucleic acid specific for lysine. *Enzymes:* Lysine:tRNA ligase (EC 6.1.1.6); Valine:tRNA ligase (EC 6.1.1.9).

with Nbs_2 was carried out at room temperature in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA. The release of 2-nitro-5-mercaptobenzoate was followed spectrophotometrically by measuring the absorbance at 412 nm with a Gilford 240 spectrophotometer against a blank without enzyme. A molar absorption coefficient of 13 600 M^{-1} cm^{-1} for 2-nitro-5-mercaptobenzoate [13] was used for the calculations in this paper.

Assay of enzyme activity. The activities of the enzymes were determined in two ways. The amino acid-dependent exchange of [^{32}P]-pyrophosphate with ATP was assayed essentially as described by Stern and Mehler.[14] The ability of the enzymes to catalyze the incorporation of ^{14}C-amino acids into tRNA was assayed as described earlier.[8,15]

Transfer of amino acid to tRNA. For the formation of the aminoacyladenylate – enzyme complexes 1 to 5 nmol of enzyme were incubated 10 min at 0 °C with 0.3 mM ^{14}C-L-amino acid (10 mCi/mmol), 1 mM ATP, 10 mM $MgCl_2$ and 0.05 M potassium phosphate buffer, pH 7.5, in a final volume of 0.2 ml. EDTA was then added to a concentration of 0.05 M, followed, in some reaction mixtures, by the addition of Nbs_2 to a final concentration of 15 mM. After 30 min at room temperature the aminoacyladenylate – enzyme complexes were isolated by chromatography on Sephadex G-25.[15] The isolated complexes were incubated at 0 °C with a 200 fold molar excess of unfractionated tRNA from yeast or *E. coli* in 2 to 3 ml of a solution containing 0.25 M ammonium sulfate, 0.01 M sodium cacodylate buffer, pH 7.0, and 0.05 M EDTA. Samples were withdrawn from the reaction mixture at different time intervals and analyzed for the formation of [^{14}C]aminoacyl-tRNA as described previously.[8,15]

Analytical sedimentation studies. A Spinco model E ultracentrifuge equipped with the RTIC temperature control unit was used for the sedimentation velocity experiments. The centrifuge was operated at 20 °C at a speed of 42 040 rpm. A plain and a wedge 12 mm double sector cell were used for the two-cell schlieren studies. The experiments were performed in 0.1 M potassium phosphate buffer, pH 7.0, at protein concentrations from 3 to 5 mg/ml.

Circular dichroism measurements were carried out in a Cary 60 recording spectropolarimeter with the Cary 6002 circular dichroism accessory attachment using a 1 mm cell. Instrument performance was checked by using the spectrum of camphorsulfonic acid as a reference.[16] The measurements were performed at room temperature in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 mM dithiothreitol. Protein concentrations were from 1 to 2 mg/ml.

RESULTS

Reaction of enzymes with Nbs_2. The reactivity of the SH-groups of the enzymes under different conditions was examined by titration with Nbs_2, as described in Methods. The enzymes were analyzed in the presence and absence of substrates and under denaturing conditions. Details of the experimental procedures are given in the legends to Fig. 1 and Table 1.

In the native lysine:tRNA ligase from yeast 13 SH-groups (13.2 ± 0.4, mean value ± standard deviation) per enzyme molecule reacted with Nbs_2. The kinetics of the reaction suggested that 2 groups reacted rapidly followed by a slower reaction of the other SH-groups (Fig. 1). Under denaturing conditions 18 SH-groups (17.5 ± 0.3) per molecule were titratable. When the enzyme was titrated in the presence of ATP or under conditions where the lysylade-

Table 1. Reactivity to Nbs_2 of SH groups of three amino acid:tRNA ligases in the presence of substrates or under denaturing conditions. Determination of SH-groups was performed as described in Methods. The incubation mixtures contained in a final volume of 0.2 ml: potassium phosphate buffer pH 7.5 (0.1 M), EDTA (0.1 mM), enzyme (5.0 – 8.0 μM), Nbs_2 (1 – 2 mM) and the additions indicated in the table. The additions were present at the following concentrations: ATP (10 mM); $MgCl_2$ (10 mM); substrate amino acid (10 mM); cognate tRNA (about 0.1 mM); guanidine.HCl (6 M).

Additions	SH-groups/enzyme (mol/mol)		
	E^{Lys}(yeast)	E^{Val}(yeast)	E^{Lys}(*E. coli*)
None	13.2	9.7	1.2
ATP	13.6	6.2	0.8
Substrate amino acid	15.0	8.9	1.1
ATP, amino acid, Mg^{2+}	13.0	8.7	0.6
Cognate tRNA	8.7	7.4	0.6
Guanidine.HCl	17.5	11.7	2.6

nylate – enzyme complex was formed, there was no change in the number of accessible SH-groups compared to the reaction in the absence of substrates (Table 1). L-Lysine alone had a small effect on the accessibility of the SH-groups for Nbs_2. A pronounced effect was obtained when the enzyme was preincubated with $tRNA^{Lys}$ from yeast. Under these conditions about 5 SH-groups were protected from reaction with Nbs_2.

When the native valine:tRNA ligase from yeast was titrated with Nbs_2 10 SH-groups (9.7 ± 0.5) per molecule of enzyme reacted (Fig. 1). Two groups reacted rapidly followed by a slower reaction of the other SH-groups. Under denaturing conditions about 12 SH-groups (11.7 ± 0.2) per enzyme molecule reacted completely in less than 1 min. When the enzyme was titrated in the presence of ATP 4 SH-groups were protected from reaction with Nbs_2 as compared to the native enzyme in the absence of substrates (Table 1). Reactions performed in the presence of L-valine or under conditions where the valyladenylate – enzyme complex was formed, gave about the same number of SH-groups accessible to Nbs_2 as did the native enzyme. Preincubation of the enzyme with $tRNA^{Val}$ from yeast protected 2 SH-groups from reaction with Nbs_2.

In the native lysine:tRNA ligase from *E. coli* 1.2 SH-group per molecule reacted with Nbs_2 while 2.6 reacting SH-groups per molecule were

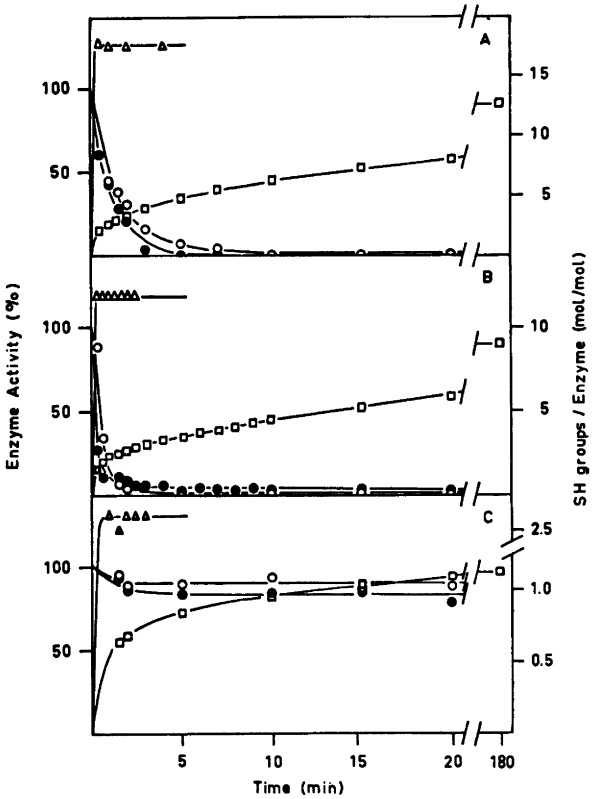

Fig. 1. Titration of SH-groups in three amino acid:tRNA ligases with Nbs_2 under native and denaturing conditions. (*A*) Lysine:tRNA ligase from yeast. (*B*) Valine:tRNA ligase from yeast. (*C*) Lysine:tRNA ligase from *E. coli*. During titrations of native enzymes ($4.0 - 9.0$ μM) with Nbs_2 ($0.05 - 0.9$ mM) aliquots were withdrawn at the times indicated and analysed for enzyme activity. □, titration under native conditions; △, titration in the presence of 6 M guanidine.HCl. Enzyme activity was determined as $[^{32}P]PP_i$ – ATP exchange (○), or incorporation of ^{14}C-amino acids into tRNA (●).

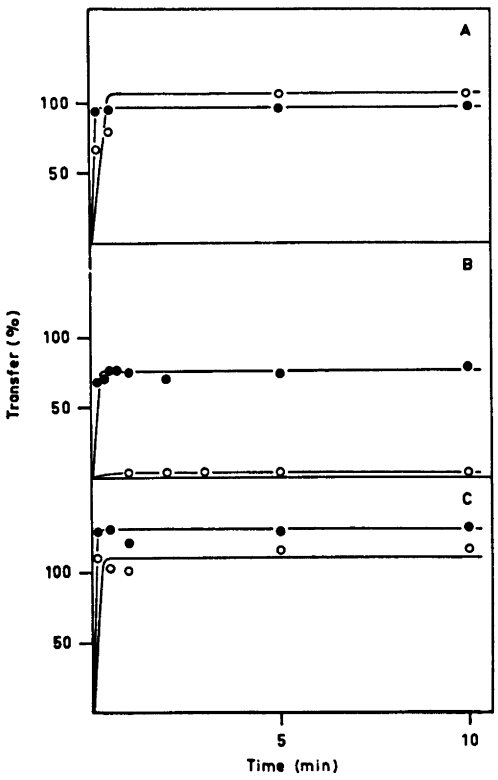

Fig. 2. Effect of Nbs_2 on the transfer of amino acid from aminoacyladenylate – enzyme complex to tRNA. Experimental procedure and conditions were as described in Methods. The transfer of amino acid to tRNA was studied with aminoacyladenylate – enzyme complexes which had been incubated with (○) or without (●) Nbs_2. (*A*) Lysine:tRNA ligase and tRNA from yeast. (*B*) Valine:tRNA ligase and tRNA from yeast. (*C*) Lysine:tRNA ligase and tRNA from *E. coli*.

found under denaturing conditions. Incubation with ATP or L-lysine did not significantly change the Nbs_2 titration curve from that obtained with the native enzyme. The presence of $tRNA^{Lys}$ from *E. coli* or incubation under conditions where the lysyladenylate – enzyme complex was formed prevented 0.6 SH-groups from reacting with Nbs_2.

Effect of Nbs_2 on enzyme activity. The amino acid:tRNA ligases were incubated with Nbs_2 as described in Methods and the legend to Fig. 1. At time intervals indicated in the figure aliquots of the reaction mixtures were removed and were assayed for enzyme activity using both the ATP – [^{32}P]pyrophosphate exchange and the tRNA acylation assay.

Lysine:tRNA ligase from yeast lost its activity somewhat faster than the rate at which 2-nitro-5-mercaptobenzoate was liberated (Fig. 1). When 5 SH-groups per molecule had reacted with Nbs_2 the enzymatic activity was completely lost.

Valine:tRNA ligase from yeast was very rapidly inactivated during the reaction with Nbs_2 (Fig. 1). A complete loss of activity was seen after blocking the two fast reacting SH-groups.

Lysine:tRNA ligase from *E. coli* retained 80 to 90 % of its activity after treatment with Nbs_2 in agreement with previous reports of its lack of sensitivity to SH-reagents.[17]

Effect of Nbs_2 on the transfer of amino acid to tRNA. The ligases were mixed with amino acid, ATP and Mg^{2+} to form their respective aminoacyladenylate – enzyme complexes. Nbs_2 was added to the reaction mixtures and the incubations were continued at room temperature for 30 min. The aminoacyladenylate – enzyme complexes were then isolated by chromatography on Sephadex G-25 as previously described.[15] Essentially the same recoveries were obtained as in parallel incubations without Nbs_2. The isolated complexes were incubated with unfractionated tRNA from yeast and *E. coli*, respectively, and the transfer of ^{14}C-amino acid from the complex to tRNA was studied (Fig. 2). The transfer of [^{14}C]valine from the complex to tRNA was completely inhibited by the incubation of the valyl-adenylate – enzyme complex with Nbs_2. The results obtained with the lysine:tRNA ligases from yeast or *E. coli*, on the other hand, showed no significant effect on the transfer reaction by the treatment of the lysyladenylate – enzyme complexes with Nbs_2.

Sedimentation properties of Nbs_2-treated enzymes. The methionine:tRNA ligase from *E. coli* and the tryptophan:tRNA ligase from beef pancreas have been shown to dissociate into subunits and at the same time lose their catalytic activities after reaction with Nbs_2.[18–20] When the lysine:tRNA ligases from yeast or *E. coli*, which also contain subunits, were reacted with Nbs_2 as described in the legend to Table 2, no dissociation into subunits occurred as shown by the lack of major changes

Table 2. Influence of Nbs_2 on sedimentation properties of the lysine:tRNA ligases from *E. coli* and yeast, with and without substrates. Sedimentations were as described in Methods. Nbs_2 and substrates, as indicated in the table, were added at the following concentrations: Nbs_2 (10 mM), $MgCl_2$ (10 mM), ATP (1 mM), L-lysine (1 mM). In the experiments with Nbs_2 the reactions were allowed to proceed for 30 min before centrifugation in order to insure complete titration of all available SH-groups.

Exp.	Enzyme	Additions	s_{obs} (10^{-13} s)
I	E^{Lys}(yeast)	–	6.0
		Nbs_2	5.5
II	E^{Lys}(yeast)	ATP, L-lysine, Mg^{2+}	5.9
		ATP, L-lysine, Mg^{2+}, Nbs_2	5.5
III	E^{Lys}(*E. coli*)	–	5.1
		Nbs_2	5.1
IV	E^{Lys}(*E. coli*)	ATP, L-lysine, Mg^{2+}	5.3
		ATP, L-lysine, Mg^{2+}, Nbs_2	5.3

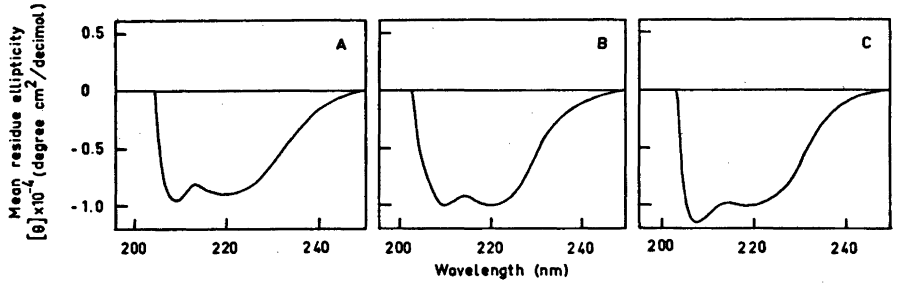

Fig. 3. Circular dichroism spectra of three amino acid:tRNA ligases. Experimental conditions were as described in Methods. The mean residue weights (MRW) were calculated from the amino acid compositions of the enzymes.[9] (*A*) Lysine:tRNA ligase (yeast), MRW = 115. (*B*) Valine:tRNA ligase (yeast), MRW = 114. (*C*) Lysine:tRNA ligase (*E. coli*), MRW = 113.

of the sedimentation coefficients of the enzymes (Table 2). Incubation of the enzymes under conditions where the lysyladenylate – enzyme complexes were formed, with or without subsequent treatment with Nbs_2, did not change the sedimentation behaviour of the enzyme – substrate complexes to the extent expected for a change of the quaternary structure of the enzymes.

Circular dichroism measurements. CD might be used to demonstrate conformational changes of enzymes caused by the interaction with substrates. Proteins in general exhibit negative circular dichroic bands around 208 and 222 nm corresponding to their helical content.[21] In the region around 220 nm tRNA shows scarcely any ellipticity.[22] This has been used by Ohta *et al.* to show a change in the helical content of tyrosine:tRNA ligase from yeast on binding its cognate tRNA.[23]

The CD of lysine:tRNA ligase from yeast and *E. coli* and valine:tRNA ligase from yeast displayed typical protein patterns with minima around 208 and 220 nm (Fig. 3). Since the circular dichroic contribution of all ligands to be tested was at a minimum at 220 nm this band was chosen to illustrate a possible change in the helical content of the enzymes when interacting with their substrates.

The enzymes were mixed with substrates at concentrations chosen so as to give a complete saturation of the enzymes. When the circular dichroism patterns were corrected for the contributions of the substrates the resultant patterns in all cases had the general appearance of that of the enzyme alone. Although, in

some cases, the difference between the ellipticity at 220 nm of the native enzyme and the ellipticity in the presence of substrate was statistically significant, the difference never exceeded 10 % of the value observed for the native enzyme.

DISCUSSION

When the three amino acid:tRNA ligases studied (lysine ligase from *E. coli* and yeast and valine ligase from yeast) were titrated with the sulfhydryl reagent Nbs_2 under denaturing conditions, the number of sulfhydryl groups found closely approximates the number of halfcystine residues determined with the amino acid analyzer.[9] This might indicate that the enzymes do not contain any disulfide bridges to stabilize their tertiary or quaternary structure. In agreement with this is the observation, that the lysine ligases dissociated into subunits when they were treated with 1 % SDS in the absence of a reducing agent (unpublished experiments).

When the three ligases were titrated with Nbs_2 under nondenaturing conditions, both the yeast enzymes were completely inactivated. The activity of the lysine ligase from *E. coli* was resistant to Nbs_2 in agreement with previous reports of its lack of sensitivity to sulfhydryl reagents.[17] There was a difference between the inactivation curves for the lysine and valine enzymes from yeast inasmuch as the valine enzyme lost its activity, measured both as ATP-pyrophosphate exchange and tRNA acylation, after the titration of the two SH-groups which reacted faster than the rest. The lysine ligase, on the other hand, showed a more gradual loss of activity, which did not parallel the titration of the two fast reacting groups, and which was not complete until about 5 SH-groups had reacted with Nbs_2. Furthermore, when the valyladenylate – enzyme complex was isolated by chromatography on Sephadex after treatment with Nbs_2, it had completely lost its ability to transfer valine to $tRNA^{Val}$, while treatment of the lysyladenylate – enzyme complexes of the two lysine enzymes had no effect on their ability to transfer lysine to $tRNA^{Lys}$. These results indicate, that for the valine ligase from yeast sulfhydryl groups are essential both for valine activation and aminoacylation of $tRNA^{Val}$. The two fast reacting sulfhydryl groups would seem to be of particular importance for the valine activation reaction.

The results indicate that the lysine ligase from yeast also requires SH-groups for the amino acid activation reaction. On the other hand, our data do not permit any conclusions regarding the possible role of SH-groups in the aminoacylation of $tRNA^{Lys}$, since one or more of the SH-groups, which were not available to Nbs_2 in the lysyladenylate – enzyme complex, could conceivably participate in the aminoacylation reaction Therefore, our results do not necessarily contradict the catalytic reaction mechanism proposed by McElroy *et. al.*[24] involving an intermediate thioacylenzyme.

One of the aims of this study was to find evidence for conformational changes of the enzymes upon binding of substrates. The results of the SH-titrations in the presence of substrates might indicate that the binding of a ligand in some cases induces conformational changes of the enzymes leading to changes in the reactivity of the sulfhydryl groups towards Nbs_2 (Table 1). The protective effect of some of the ligands could also be explained by a direct mechanism where an SH-group in close proximity of the substrate binding site is prevented from reacting with Nbs_2 by the presence of the substrate. The changes in hydrodynamic properties or circular dichroism recorded for these enzymes in the presence of substrates are too small to warrant any conclusions regarding possible alterations of their secondary or tertiary structure induced by the ligands.

Acknowledgements. This work was supported by the Swedish Cancer Society.

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Received September 13, 1976.