Intermediate Reactions in the Binding of Aminoacyl-Transfer Ribonucleic Acid to Rat Liver Ribosomes

THE INTERACTION OF CHOLESTERYL 14-METHYLHEXADECANOATE

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1. Transferase I from rat liver extracted with iso-octane binds significantly less aminoacyl-tRNA than the non-extracted enzyme. The original activity can be fully restored by the addition of cholesteryl 14-methylhexadecanoate. The binding capacity for GTP is not affected by the extraction. 2. In the presence of extracted transferase I the binding of aminoacyl-tRNA to ribosomes is decreased to 11–26% and the simultaneous binding of GTP to 32–43%. Cholesteryl 14-methylhexadecanoate induces a full reactivation of the extracted enzyme in both respects. 3. Extracted complexes A (aminoacyl-tRNA–GTP–transferase I) become bound to ribosomes to the same extent as the corresponding non-extracted preparations. 4. It is concluded that cholesteryl 14-methylhexadecanoate interacts with the binding site of transferase I for aminoacyl-tRNA and secondarily with that for GTP. It does not affect the binding site for ribosomes.

Cholesteryl 14-methylhexadecanoate affects the activity of some enzymes required for protein synthesis in rat liver *in vitro*. This is true not only for enzymes present in the cell sap, such as aminoacyl-tRNA synthetases and peptide-elongation factors (Hradec & Dušek, 1968) but also for RNA polymerase of liver cell nuclei (Komárková & Hradec, 1971). Evidence has been obtained that this ester is a constituent of the enzyme molecules that is essential for their normal function. Aminoacyl-tRNA ligases from various sources showed significantly decreased activities after the extraction of this compound from enzyme preparations (Hradec & Dušek, 1969). The usual enzymic activity was fully restored by the addition of appropriate quantities of cholesteryl 14-methylhexadecanoate. Essentially the same results were obtained with peptide elongation factors from human tonsils and rat liver (2.20–4.26 nmol/mg of protein extracted with different solvents) (Hradec *et al.*, 1971). It seems that the reactivating effect of cholesteryl 14-methylhexadecanoate is specific for this particular ester since cholesteryl esters with different acid moieties were not able to affect the activity of aminoacyl-tRNA synthetases (Hradec & Sommerau, 1970).

For the elucidation of mechanisms by which this compound affects the enzymic activity, more thorough studies on the interaction of this ester with active centres of enzymes are desirable. Multifunctional enzymes with more active centres seem to be a model suitable for such studies.

One of the peptide-elongation factors, the binding enzyme (McKeenan & Hardesty, 1969) or trans-

ferase I (Schneir & Moldave, 1968) is such a typical multifunctional enzyme involved in protein synthesis. This factor catalyses the binding of aminoacyl-tRNA to the ribosome (Ibuki & Moldave, 1968). During this multi-step reaction the binding enzyme combines with aminoacyl-tRNA, GTP and the ribosome to form intermediate complexes and has apparently separate binding sites for aminoacyl-tRNA, GTP and the ribosome (Shorey *et al.*, 1969). Further, there seems to be a regulatory site for the binding of nucleotides (Lin *et al.*, 1969). Evidence has been presented (Hradec, 1972*a,b*) that the individual binding sites of transferase I show a relatively low interdependence. Transferase I combines with aminoacyl-tRNA to form complex A (Hradec, 1972*a*), with GTP (complex B) or with both these compounds together to form complex C (Hradec, 1972*b*).

In the experiments described in the present paper cholesteryl 14-methylhexadecanoate was removed from the binding enzyme by extraction with an organic solvent. The ability of such extracted preparations to form different complexes involved in the binding reaction was then tested to see if all or only some particular binding sites of transferase I interact with cholesteryl 14-methylhexadecanoate.

Materials and Methods

The binding enzyme from rat liver was purified and extracted with iso-octane as described by Hradec *et al.* (1971). All methods used for the assay of transferase I complex-formation with aminoacyl-tRNA

and GTP as well as for the binding of these complexes to ribosomes were described by Hradec (1972a,b). In most experiments a mixture of [^3H]GTP and [$\gamma\text{-}^{32}\text{P}$]GTP was used to determine the amount of GTP that had been split to GDP during the reaction.

Complexes of transferase I with aminoacyl-tRNA (complex A) and the ternary complex C were isolated by the second procedure of Hradec (1972a). Both non-extracted and extracted enzymes were used for the preparation of complexes. Where indicated, complexes were prepared by using non-extracted transferase I and were extracted afterwards with the organic solvent. In such a case, immediately after the adsorption of complexes on Whatman DE-20 filter-paper discs, the filters were quickly frozen on solid CO_2 and freeze-dried. These dried complexes were then extracted with iso-octane by the usual procedure (Hradec *et al.*, 1971). After the extraction, complexes adsorbed on filter-paper discs were quickly dried *in vacuo* and eluted from the paper as described by Hradec (1972a). Control preparations of transferase I complexes were treated in the same way, but the extraction step was omitted.

Results

Complex-formation with extracted binding enzymes

The extracted preparations of the binding enzyme had a significantly decreased activity for binding of aminoacyl-tRNA (Fig. 1). This decrease was very similar to the decrease in the binding of aminoacyl-tRNA to ribosomes in systems that contain extracted transferase I (Hradec *et al.*, 1971). A normal activity for formation of complex A with extracted transferase I could be obtained when cholesteryl 14-methylhexadecanoate was added to incubation mixtures in quantities corresponding at least to those that had been removed by the extraction (4.05–4.31 pmol/mg of protein). Lower amounts than these showed a significant inhibitory effect on the residual activity of transferase I (Fig. 2).

The combination of extracted binding enzymes with GTP was not affected by the extraction. With some extracted enzyme preparations even a slightly higher binding of this nucleotide was observed when compared with non-extracted enzymes. Extracted preparations also showed a very similar response to increasing amounts of GTP in the incubation mixture, as did the control non-extracted enzyme. Cholesteryl 14-methylhexadecanoate had no effect on the binding of GTP to transferase I with either non-extracted or extracted preparations.

Extraction of the binding enzyme significantly decreased its ability to form complex C (combination with both aminoacyl-tRNA and GTP). The decrease in binding of aminoacyl-tRNA to extracted enzyme

Fig. 1. Formation of complex A (Hradec, 1972a) with extracted and non-extracted transferase I preparations

Incubation mixtures contained 50mM-tris-HCl buffer, pH 7.5, 6mM-MgCl₂, 50mM-KCl, 262 pmol of [^3H]phenylalanyl-tRNA and the indicated quantities of the binding enzyme in a final volume of 0.1 ml. Both enzyme preparations (---, extracted; —, non-extracted) were of the same batch. The incubation was done as described by Hradec (1972a).

preparations corresponded approximately to that obtained in the formation of complex A. However, the binding of GTP to complex C was significantly less inhibited by the extraction than was the binding of aminoacyl-tRNA (Fig. 3). After the addition of cholesteryl 14-methylhexadecanoate in quantities that corresponded to those removed by the extraction into incubation mixtures with extracted transferase I preparations, the rate of formation of complex C corresponded to that with the non-extracted control enzyme (Fig. 4). Small quantities of the ester again had a slight inhibitory effect on the residual activity of extracted transferase I; however, this was much less significant than with the formation of complex A.

Complex A prepared from extracted binding enzyme binds only slightly less GTP than the corresponding complex of non-extracted enzyme. The normal combination of this complex with the nucleotide could be obtained after the addition of cholesteryl 14-methylhexadecanoate to incubation mixtures. In this case, the system was apparently very sensitive to the quantity of the ester added and addition of larger amounts than those extracted showed a significant inhibitory effect.

Fig. 2. Effect of cholesteryl 14-methylhexadecanoate on the binding of aminoacyl-[^{32}P]tRNA by an extracted transferase I preparation

Incubation mixtures contained in a final volume of 0.1 ml: 50 mM-tris-HCl buffer, pH 7.5, 6 mM-MgCl₂, 50 mM-KCl, 3.8 μg of transferase I protein, 0.32 pmol of aminoacyl-[^{32}P]tRNA and cholesteryl 14-methylhexadecanoate as indicated; the mixtures were incubated as described by Hradec (1972a). The non-extracted transferase I of the same batch bound 0.296 pmol of aminoacyl-[^{32}P]tRNA. Its activity was not significantly affected by the addition of cholesteryl 14-methylhexadecanoate in the quantities used with the extracted enzyme.

Binding of aminoacyl-tRNA to ribosome with extracted transferase I and its complexes

In systems that contained ribosomes, [^{14}C]-phenylalanyl-tRNA, [^3H]GTP and [$\gamma\text{-}^{32}\text{P}$]GTP and extracted binding enzyme, a significantly decreased binding of both aminoacyl-tRNA and GTP to ribosomes was found. However, the decrease in binding of GTP was not proportional to that of aminoacyl-tRNA (Fig. 5). Thus with different preparations of extracted transferase I only 11–26% of phenylalanyl-tRNA became bound to ribosomes when compared with control non-extracted enzymes, whereas the binding of GTP was decreased only to 32–43%, when compared with the activity of the corresponding non-extracted enzyme (taken as 100%). Values close to those found with corresponding non-extracted enzyme preparations were obtained after the addition of cholesteryl 14-methylhexadecanoate to incubation mixtures.

When complex A prepared from extracted enzyme

Fig. 3. Formation of complex C (Hradec, 1972b) with non-extracted and extracted transferase I

Incubation mixtures contained 50 mM-tris-HCl buffer, pH 7.5, 6 mM-MgCl₂, 50 mM-KCl, 0.2 mM-GTP (100 000 c.p.m. of [^3H]GTP and 250 000 c.p.m. of [$\gamma\text{-}^{32}\text{P}$]GTP) and 200 pmol of [^{14}C]phenylalanyl-tRNA in a final volume of 0.1 ml; the mixtures (---, extracted; —, non-extracted) were incubated as described by Hradec (1972b). ●, ^3H ; ■, ^{14}C ; ▲, ^{32}P .

was used the same binding of the aminoacyl-tRNA moiety of the complex to ribosomes was obtained as with complexes prepared from non-extracted enzymes. Complex A prepared from non-extracted transferase I and extracted with iso-octane after the isolation was bound to ribosomes to the same extent as the non-extracted preparation.

Both the aminoacyl-tRNA and GTP moieties of complex C prepared with extracted binding enzymes became bound to ribosomes to the same extent as the corresponding moieties of complex C prepared with non-extracted transferase I. The same results were obtained with complex C prepared from non-extracted enzymes and extracted after the isolation.

When extracted transferase I preparations were preincubated with GTP, and aminoacyl-tRNA and ribosomes were added after such a preincubation period, significantly less aminoacyl-tRNA became bound to ribosomes. However, the binding of GTP was only slightly inhibited, and it was significantly less than in systems that were not preincubated (Fig. 6). A complete reactivation of the extracted enzyme

Fig. 4. Effect of cholesteryl 14-methylhexadecanoate on the binding of phenylalanyl-tRNA and GTP by an extracted transferase I preparation

The composition of incubation mixtures was the same as described in the legend for Fig. 3 except that they contained only 125000 c.p.m. of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. They contained 0.32 mg of transferase I protein. Iso-octane extracted 4.05 nmol of the ester/mg of enzyme protein from the preparation used. The values obtained with the corresponding quantity of non-extracted transferase I from the same batch were 2.1 pmol of $[\text{C}^{14}]\text{phenylalanine}$, 2480 c.p.m. of $[\text{H}^3]\text{GTP}$ and 2850 c.p.m. of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. $\circ$, ^3H ; $\square$, ^{14}C ; Δ , ^{32}P .

was again obtained with cholesteryl 14-methylhexadecanoate.

Discussion

The results presented in this paper indicate that the interaction of cholesteryl 14-methylhexadecanoate with transferase I described by Hradec *et al.* (1971) is predominantly limited to its binding site for aminoacyl-tRNA. Since complexes A and C prepared from extracted enzymes or both these extracted complexes synthesized with non-extracted enzymes are bound to ribosomes to the same extent as the control preparations, the ester has apparently no effect on the binding site of transferase I for the ribosome. The effect of this compound on the binding site for GTP is not quite clear. In the absence of aminoacyl-tRNA, GTP becomes bound to extracted enzyme with the same yields as to non-extracted preparation. How-

Fig. 5. Binding of phenylalanyl-tRNA and GTP to ribosomes in the presence of a non-extracted and extracted transferase I preparation

Incubation mixtures contained 50 mM-tris-HCl buffer, pH 7.5, 6 mM-MgCl₂, 50 mM-KCl, 50 μg of poly(U), 0.2 mM-GTP (100000 c.p.m. of $[\text{H}^3]\text{GTP}$ and 300000 c.p.m. of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$), 200 pmol of $[\text{C}^{14}]\text{phenylalanyl-tRNA}$ and 350 μg of ribosomes, in a final volume of 0.1 ml; mixtures (---, extracted; —, non-extracted) were incubated as described by Hradec (1972b). Iso-octane extracted 4.05 nmol of cholesteryl 14-methylhexadecanoate/mg of enzyme protein from the preparation used. $\bullet$, ^3H ; $\blacksquare$, ^{14}C ; $\blacktriangle$, ^{32}P .

ever, the binding of this nucleotide to complex A or to complex C is decreased, although this decrease is less significant than that of aminoacyl-tRNA binding. Evidence obtained in the present and in previous experiments (Hradec, 1972b) suggests that the combination of transferase I with GTP is regulated by the amount of aminoacyl-tRNA bound to the enzyme. This mechanism apparently operates fully only if GTP becomes bound to the enzyme simultaneously with aminoacyl-tRNA, or if it is bound by the preformed complex A. If the binding of aminoacyl-tRNA to transferase I occurs after the binding of GTP the binding of this nucleotide to ribosomes is less affected by aminoacyl-tRNA (Hradec, 1972b) and by cholesteryl 14-methylhexadecanoate. Thus it seems that the binding site of transferase I for GTP is affected by this ester not primarily, but secondarily, through the interaction of this compound with the aminoacyl-tRNA binding site.

The systems used in previous studies (Hradec & Dušek, 1969; Hradec *et al.*, 1971) were very sensitive to the amount of cholesteryl 14-methylhexadecanoate

Fig. 6. Binding of aminoacyl-tRNA to ribosome in the presence of complex B (Hradec, 1972b) formed from non-extracted and extracted transferase I

Incubation mixtures contained, in a final volume of 0.1 ml: 50 mM-tris-HCl buffer, pH 7.5, 6 mM-MgCl₂, 50 mM-KCl, 0.2 mM-[³H]GTP and 125 µg of transferase I protein; the mixtures (---, extracted; —, non-extracted) were incubated at 37°C for 10 min. After this preincubation 180 µg of ribosomes and quantities of aminoacyl-[³²P]tRNA as indicated were added and the incubation was continued for 10 min. ●, ³H; ▲, ³²P.

added to incubation mixtures, and doses that were too high had an inhibitory effect on these enzymic reactions. On the other hand, very high doses of this ester do not show any inhibitory effect on complex A

formation with extracted enzymes. However, the binding of aminoacyl-tRNA to ribosomes in the presence of extracted transferase I shows a very strict dose-dependence (Hradec *et al.*, 1971) and doses only slightly higher than optimum have an inhibitory effect. At present no satisfactory explanation can be found for this, unless an interaction of the ester with the ribosome is assumed to occur.

No binding of aminoacyl-tRNA to the ribosome is apparently possible without the prior combination of aminoacyl-tRNA with the binding enzyme (Hradec, 1972a). This reaction thus represents the limiting step of the whole binding reaction. As cholesteryl 14-methylhexadecanoate affects only this reaction step, its association with the binding enzyme may represent a regulatory mechanism in the supply of ribosomes with aminoacyl-tRNA.

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