THE LIGAND-INDUCED SOLUBILITY SHIFT IN SALTING OUT CHROMATOGRAPHY

A new affinity technique, demonstrated with phenylalanyl- and isoleucyl-tRNA synthetase from baker's yeast

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1. Introduction

Recently I have described the use of salting out chromatography with unsubstituted hydrophilic gels for the purification of proteins [1,2]. A protein fraction isolated by this procedure is characterised by the identical solubility of all its constituents. A major improvement of this method could be expected if one were able to alter specifically the solubility of one of the components of the mixture, since this single species should, on rechromatography, appear either before or after all the other proteins. Such a slight shift in solubility is expected to occur on complexation of an enzyme with its ligand, and might consequently be the basis for an improvement of salting out chromatography.

The usefulness of this principle is demonstrated for phenylalanyl- (RC 6.1.1.20) and isoleucyl-tRNA synthetase (EC 6.1.1.5), which can be subjected to salting out chromatography after complexation with the respective tRNA. Several practical and mechanistic implications are discussed.

2. Materials and methods

$tRNA^{Phe}$–C–C–A [3] and $tRNA^{Ile}$–C–C–A [4] were purified as described. All other materials and methods were identical to those in [1,2].

3. Results and discussion

3.1. *The ligand-induced solubility shift*

In fig.1 a phenylalanyl-tRNA synthetase fraction obtained by salting out chromatography [1,2] has been rechromatographed after addition of $tRNA^{Phe}$–C–C–A. The main protein peak (fraction 35–45) is followed by a smaller protein peak (fraction 63–80) of which the latter coincides with the eluted $tRNA^{Phe}$, as seen from the absorbance at 260 nm. Almost no enzymatic activity can be detected in the main peak, in which the ligand free enzyme appears (see fig.2). All the enzymatic activity now appears in the $tRNA^{Phe}$ containing peak. According to sodium dodecyl sulfate gel electrophoresis [5] the protein in this peak consists to more than 95% of the a_2b_2 system of phenylalanyl-tRNA synthetase. Only a few very minor additional bands appear, each being less than 1% of total protein.

In fig.2 an identical experiment is shown in which the amount of the $tRNA^{Phe}$ added was suboptimal with respect to the content of phenylalanyl-tRNA synthetase. Now the enzymatic activity splits into two fractions. The first appears without tRNA, the second cochromatographs with $tRNA^{Phe}$. This indicates that, indeed, stoichiometric complexes of enzyme and $tRNA^{Phe}$ are being chromatographed. The enzyme in the second case is less pure than that obtained in fig.1, because a broader fraction from the first salting out chromatography was used in this case.

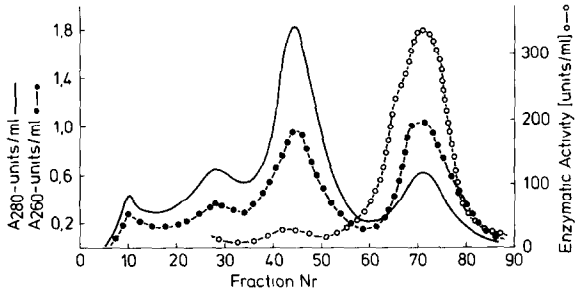

Fig.1. Solubility shift of phenylalanyl-tRNA synthetase induced by excess of $tRNA^{Phe}$–C–C–A. To 570 A_{280} units of a phenylalanyl-tRNA synthetase fraction obtained by salting out chromatography [1] and dissolved in 15 ml 0.03 M potassium phosphate buffer (pH 6.0) were added 210 A_{260} units of $tRNA^{Phe}$–C–C–A. The mixture was dialysed against a 0.01 M $MgSO_4$ containing aqueous ammonium sulfate solution saturated to 47% and adjusted to pH 6.0. After dialysis the solution was passed over a 3 × 11 cm Sepharose 4B column equilibrated with 50% saturated ammonium sulfate solution adjusted to pH 6.0. The column was developed with a gradient of aqueous 50–25% saturated ammonium sulfate solution (pH 6.0) containing 0.01 M $MgSO_4$ in twice 1 liter. Fractions, 16 ml, were collected. The position of tRNA in the elution profile can be seen from the increase in ultraviolet absorption at A_{260} nm relative to ultraviolet absorption at 280 nm. Enzymatic tests were performed as in [1].

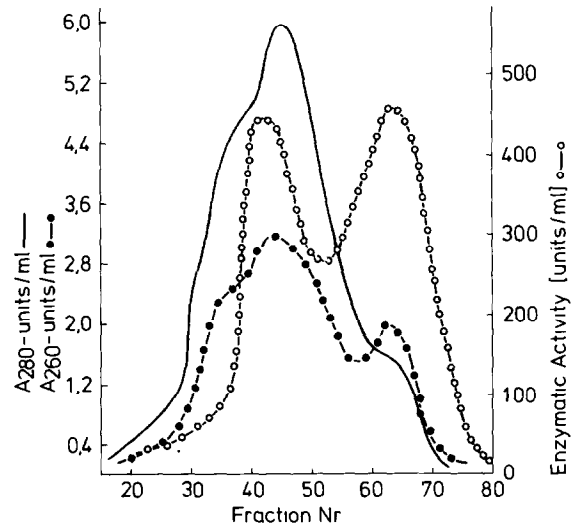

Fig.2. Solubility shift of phenylalanyl-tRNA synthetase with excess enzyme over $tRNA^{Phe}$. To 2600 A_{280} units of an analogous fraction to that used in fig.1 were added 400 A_{260} units $tRNA^{Phe}$–C–C–A. The procedure was performed as in fig.1.

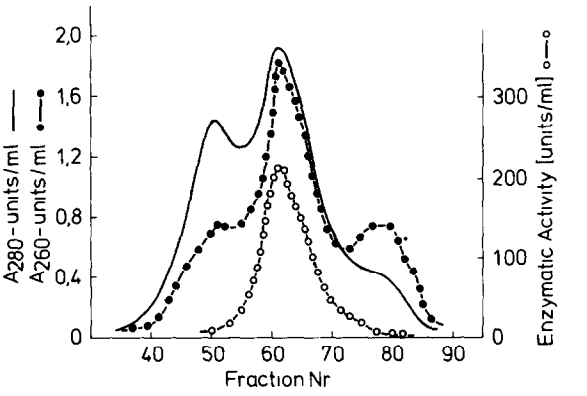

Fig.3. Solubility shift of isoleucyl-tRNA synthetase induced by excess $tRNA^{Ile}$–C–C–A. To 400 A_{280} units of an isoleucyl-tRNA synthetase fraction obtained by salting out chromatography [1] were added 300 A_{260} units $tRNA^{Ile}$–C–C–A. The procedure was performed as in fig.1.

In both examples, however, (fig.1,2) the complex is eluted at 4–4.5% lower saturation of ammonium sulfate, as compared to the free enzyme.

Figure 3 shows the results of a chromatography with an isoleucyl-tRNA synthetase fraction, which was carried out in an analogous manner to that described for phenylalanyl-tRNA synthetase in fig.1. In this case there is also an enzyme·tRNA complex formed, but, within the limits of experimental error, it is not shifted from the position where it appeared during chromatography in absence of $tRNA^{Ile}$ and no further purification is obtained [1]. Excess $tRNA^{Ile}$ is eluted after the protein (fraction 73–86). For practical purposes the isolation of a stoichiometric complex was also useful in this case. The complex could be obtained in a homogeneous state by filtration over a 50 ml Sephadex CM-50 column after dialysis against dilute buffer. Since the protein fraction used had originally been subjected to Sephadex CM-50 chromatography, according to the principles of affinity elution [5,6] all the proteins bind to Sephadex CM-50 except for the (E^{Ile}·$tRNA^{Ile}$) complex, which runs through.

From the examples given it can be seen, that at least in favourable cases a ligand-induced solubility shift may be helpful in protein purification. It remains, however, to be established whether this phenomenon is more general or whether it is a rare exception. As a

rational further approach one might suggest the use of ligand analogs bearing substituents which cause a larger solubility shift than merely the ligand itself.

3.2. *Stoichiometry of complex formation of phenylalanyl- and isoleucyl-tRNA synthetase with their respective tRNA*

From fig.1 fraction 73 it can be seen, that the ratio of A_{260}/A_{280} is 1.67 for the [phenylalanyl-tRNA synthetase·$tRNA^{Phe}$] complex. A value of 1.62 is found performing an analogous experiment with homogeneous enzyme (data not shown here). Titration experiments show that on complex formation of enzyme with tRNA no alterations in ultraviolet extinction can be observed if compared to the free components. Hence the stoichiometry of the complex can be calculated from the A_{260}/A_{280} absorption ratios of the pure components $tRNA^{Phe}$ (2.1) and enzyme (0.54) in combination with the molar extinction ($tRNA^{Phe}$ = 0.58 A_{260}/nmol, enzyme = 0.29 A_{280}/nmol) [7]. In this way a stoichiometry of 2 tRNA molecules for each enzyme molecule was determined. For a 1:1 complex an A_{260}/A_{280} ratio of 1.32 would be expected [5,8]. In an identical manner for isoleucyl-tRNA synthetase a stoichiometry of 1 tRNA per 1 enzyme molecule was determined. For the isoleucyl-tRNA synthetase, which is a single chain enzyme, this result is not unexpected (discussed in [4]). The result obtained with phenylalanyl-tRNA synthetase is however puzzling. Two independent binding sites with identical K_{ass} have been shown [9] in a low ionic strength phenylalanyl-tRNA synthetase from baker's yeast. Hence, one would expect an equilibrium mixture of free enzyme, 1:1 and 1:2 complexes of enzyme and tRNA. Within the limits of error, however, under our conditions only 1:2 complexes exist besides excess free enzyme. The situation becomes even more complicated in view of the fact that during affinity elution at low ionic strength quite the opposite is the case [8]. Here there is always free enzyme in addition to a 1:1 complex of enzyme and $tRNA^{Phe}$. Although we cannot explain the meaning of these conflicting results at the moment, I would tend to agree with the conclusion in [7] that some sort of undefined 'interprotomer cooperativity' must exist during interaction of $tRNA^{Phe}$ and phenylalanyl-tRNA synthetase.

3.3. *Specificity of complex formation between phenylalanyl-tRNA synthetase and tRNA*

On chromatography of phenylalanyl-tRNA synthetase with $tRNA^{Phe}$–C–C–A lacking the 3′-terminal adenosine, an identical solubility shift was observed as with $tRNA^{Phe}$–C–C–A. With noncognate $tRNA^{Val}$–C–C–A, however, no complex formation was observed at all. This again is conflicting with data obtained during work at low ionic strength [8,9], where a high degree of unspecific complex formation occurs. Obviously the binding forces at low and high ionic strength are different. It seems reasonable to assume that unspecific binding at low salt is due to ionic interactions of the phosphate backbone with positive charges on the enzyme. These ionic interactions are probably suppressed in high salt, where under these conditions more specific nucleoside–enzyme interactions are stabilized.

3.4. *Stability of enzyme–ligand complexes at high salt*

Phenylalanyl-tRNA synthetase at low ionic strength binds its cognate $tRNA^{Phe}$ rather tightly [8,9]. A moderate increase of ionic strength weakens the binding significantly [9]. Hence it was very surprising that stable stoichiometric complexes can be chromatographed at high concentration of ammonium sulfate. A possible explanation for this phenomenon has already been suggested above (see section 3.3).

4. Concluding remark

The method exemplified here for two aminoacyl-tRNA synthetases will be of general use only if other stable enzyme–ligand complexes also exist at high salt concentrations. This seems probable for the following reasons. X-ray crystallographic investigations have shown that ligands can be diffused into crystals and remain tightly bound to their binding sites. In addition, it has been demonstrated [10], that at 1 M antichaotropic ion, sulfate, the binding of polymer bound NAD to several dehydrogenases is significantly increased. It should also be mentioned that with a more versatile ligand than tRNA, a concentration of the ligand sufficient to saturate the enzyme may be added to the gradient used to develop the column.

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