Hypothesis

ENZYME SPECIFICITY RESULTING FROM PROOFREADING EVENTS

Friedrich VON DER HAAR

Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Göttingen, Hermann-Rein-Straße 3, FRG

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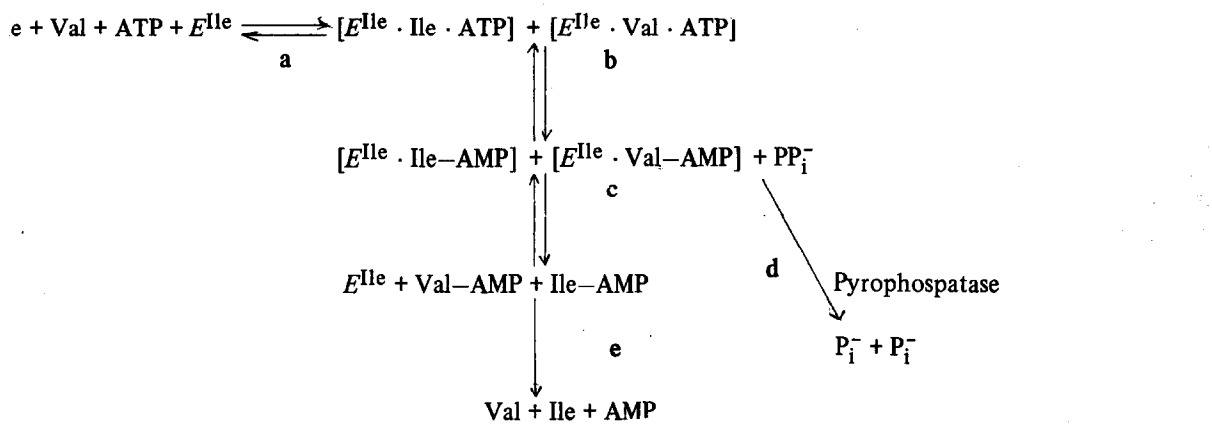
In 1974 J. J. Hopfield developed a mechanistic scheme, called 'kinetic proofreading', which was able to explain, at least partially, the high specificity required in biosynthetic processes [1]. In this concept, attention was drawn to the advantages gained by successive steps with respect to enzyme specificity. This scheme was almost immediately accepted as conceptually important, since such an explanation was particularly necessary for enzyme catalysed reactions selecting one substrate from a group of very similar metabolites such as amino acids, aminoacylated tRNA and nucleotides [2]. It was obvious that the differences in the intrinsic free energy of complex formation between an enzyme and two such similar metabolites may not always be large enough to account for the very high specificity required.

Let us consider one example. It is known that isoleucyl–tRNA synthetase [E^{Ile}] from various sources is able to misactivate valine (Scheme 1) [3,4].

$$Val + ATP + E^{Ile} \rightleftharpoons [E^{Ile} \cdot Val{-}AMP] + PP$$

Scheme 1

The subsequent transfer of the misactivated valine to $tRNA^{Ile}$–C–C–A could, however, never be observed; instead valine was liberated from the [E^{Ile} · Val–AMP]complex after addition of $tRNA^{Ile}$–C–C–A [3]. This example was taken by Hopfield as a typical case in which kinetic proofreading might function. In terms of kinetic proofreading the mechanism is controlled as shown in Scheme 2.



Scheme 2

According to this scheme specificity is enhanced by making use twice of the Michaelis kinetic ability to distinguish between valine and isoleucine (equilibria **a** and **c** in the scheme), thus resulting in an error rate f_0^2 instead of f_0 obtained by only a single discrimination. An essential prerequisite for kinetic proofreading is that pyrophosphate is removed by pyrophosphatase via pathway **d** in order to keep the back reaction in equilibrium **b** at an almost negligable level. Otherwise the population of aminoacyl-adenylate · enzyme complexes would be governed by the levels of the amino acid · enzyme complexes and a two-fold discrimination would not be possible. Hopfield states that this prerequisite is achieved at the expense of energy (in this case splitting of PP). According to Scheme 2, any event removing a product of an early step from the overall equilibrium would lead to the same result, irrespective of whether energy is spent or gained. Hence the assumption that the organism has to pay with energy for the increase of specificity [1] is not necessarily correct. In fact, however, in all examples cited by Hopfield, energy is spent via pathway **d** [1]. Another prerequisite is, that the equilibrium **b** is obtained by free dissociation and association of the aminoacyl-adenylates. From what is known in the literature this has to be doubted [3–5]. In a later investigation Hopfield attempted to verify his hypothesis experimentally [6]. In order to determine the amount of amino acid transferred from the [enzyme · aminoacyl–AMP] complex to $tRNA^{Ile}$, he added excess of elongation factor Tu to the reaction mixture [6]. Tu is known to complex aminoacyl–tRNA very tightly thus protecting the labile aminoacyl–tRNA ester linkage from any hydrolytic event [7] (Scheme 3)

Measuring in addition the amount of AMP generated from ATP during this transfer experiment, he found that for one Val–$tRNA^{Ile}$ complexed to Tu, 270 AMP were generated, whereas for one Ile–$tRNA^{Ile}$ complexed to Tu, 1.6 AMP were formed. From this he concluded that for any [E^{Ile} · Val–$tRNA^{Ile}$] formed, 270 valine molecules were activated, consuming an equivalent number of ATP molecules. Hence he concluded that one of the prerequisites of kinetic proofreading, which is energy consumption to prevent misacylation, was fulfilled by this experimental result.

At about the same time we investigated this system by an entirely different approach [4,8]. We used a number of $tRNA^{Ile}$ with modified 3′-terminal adenosine – the reactive part of tRNA – to get insight into the chemistry of transacylation and prevention of mistransacylation. This investigation led us to the conclusion that:

(a) Every misactivated valine is transiently transferred to $tRNA^{Ile}$ (Scheme 4, **b**).

(b) The resulting misacylation is corrected by hydrolysis of the Val–$tRNA^{Ile}$ ester linkage, before the wrong product is released from [E^{Ile} · Val–$tRNA^{Ile}$] complex (Scheme 4, **c**).

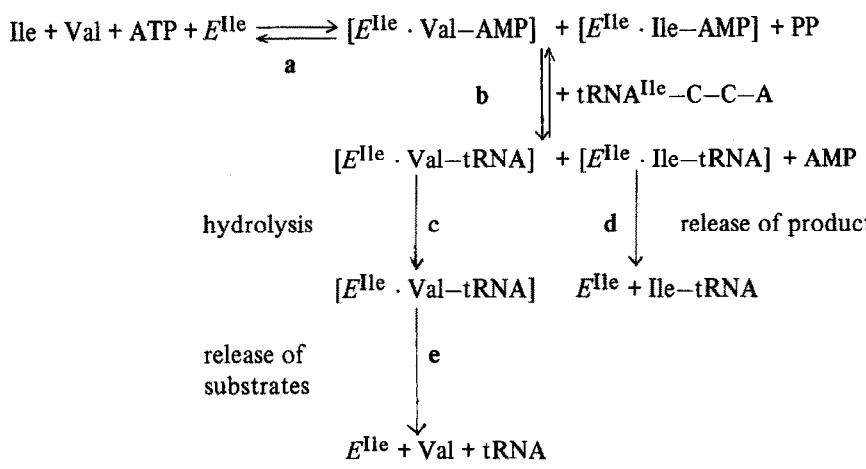
Transient transfer of threonine misactivated by valyl–tRNA synthetase to $tRNA^{Val}$ was demonstrated using rapid quenching techniques by A. Fersht and M. Kaethner [9]. We later investigated the misactivation of threonine by valyl–tRNA synthetase using our approach of modified acceptor tRNA [10]. Our data agreed entirely with that of A. Fersht [9] and this system showed a very similar behaviour to the misactivation of valine by isoleucyl–tRNA synthetase [8,10].

Hence, the complex [E^{Ile} · Val–$tRNA^{Ile}$] is an obligatory intermediate during the reaction pathway leading to correction of misactivation by isoleucyl–tRNA synthetase. Therefore for every valine transferred only one ATP is consumed. Consequently the basic feature of 'kinetic proofreading', to consume many ATP molecules in order to prevent misvalylation of $tRNA^{Ile}$ (Scheme 2), is not fulfilled in this

$$[E^{Ile} \cdot \text{Val–AMP}] \xrightarrow{tRNA^{Ile}} \text{Val–}tRNA^{Ile} + \text{AMP} + E^{Ile} \xrightarrow{\text{Tu}} [\text{Tu} \cdot \text{Val–}tRNA^{Ile}]$$

$$[E^{Ile} \cdot \text{Ile–AMP}] \xrightarrow{tRNA^{Ile}} \text{Ile–}tRNA^{Ile} + \text{AMP} + E^{Ile} \xrightarrow{\text{Tu}} [\text{Tu} \cdot \text{Ile–}tRNA^{Ile}]$$

Scheme 3



Scheme 4

particular case and the correction cannot follow the 'kinetic proofreading' scheme. One then has to ask, what do the experimental results obtained by addition of Tu to the misvalylation assay mean [6]. Obviously by this procedure only those Val–$tRNA^{Ile}$ species were determined, which escaped the hydrolytic correction reaction. This is in itself an important figure to know.

Since we were able to show, that the misactivation of valine was corrected by a hydrolytic event, which is most probably independent of the activation process, we named this mechanism 'chemical proofreading'. Generally 'kinetic' as well as 'chemical' proofreading makes use of consecutive steps to enhance specificity; they differ, however, in the way this goal is achieved. Thus it seems worthwhile to define in an unequivocal manner the conceptual difference between 'kinetic' and 'chemical' proofreading.

As stated above, kinetic proofreading potentiates the intrinsic differences in free energy between similar substrates and a single enzyme. Hence in a population of substrates with

$$K_{dissA} < K_{dissB} < K_{dissC} < K_{dissD}$$

kinetic proofreading will always select for the substrate with the lowest K_{diss}. The real existence of the kinetic proofreading mechanism remains to be established. Recently it was reported that codon/anticodon recognition specificity might be enhanced by kinetic proofreading [11]. The energy necessary in this case was attributed to the hydrolysis of GTP. The data given are too preliminary to finally establish whether this process fulfills all the requirements of kinetic proofreading. However, the assay system described in that report will eventually allow one to come to a final decision.

Chemical proofreading on the other hand depends on a defined enzyme catalysed reaction based on specificity criteria entirely independent of the free energy of complex formation. Therefore the above mentioned basic restriction of kinetic proofreading to select always for the substrate with lowest K_{diss} does not exist. One can easily imagine a case, in which via chemical proofreading a substrate with a higher K_{diss} is selected against a second substrate with lower K_{diss}. This would of course not be very efficient with respect to product formation if both substrates are present in about equimolar amounts. Such a case might however be advantageous if a weakly bound substrate which is present in a high concentration has to be selected against a strongly bound substrate which is present in only small amounts. The existence of a 'chemical proofreading' process has been established in two cases [8–10].

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