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Metabolite channeling versus free diffusion: reinterpretation of aldolase-catalysed inactivation of glyceraldehyde-3-phosphate dehydrogenase

Christen *et al.* (1976) showed that when aldolase (fructose-1,6-bisphosphate aldolase, EC 4.1.2.13) acted on fructose 1,6-bisphosphate in the presence of $Fe(CN)_6^{3-}$, the *P*-O-CH_2-CO-CHO (hydroxypyruvaldehyde phosphate, HPAP) formed, a powerful arginine-modifying reagent (Patthy, 1978), inactivated the parent aldolase by destroying a particular arginine residue in its active site (Patthy *et al.*, 1979). Since this reagent is rapidly hydrated to become unreactive, Patthy & Vas (1978) used it to investigate the possibility of substrate channeling from aldolase to glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; GAPDH), as proposed by Ovádi & Keleti (1978) and Orosz & Ovádi (1987). When GAPDH was also present during the occurrence of the above aldolase-catalysed reaction, inactivation of the former enzyme has been observed. The plot of number of GAPDH molecules inactivated per catalytic cycle of aldolase against GAPDH concentration could best be explained by the formation of a 1:1 aldolase–GAPDH complex with an apparent dissociation constant of about 10^{-5} M (Patthy & Vas, 1978). The hypothesis of direct transfer of the reagent between the enzymes in such a complex was supported by the fact that much less GAPDH was inactivated when it was added to the reaction mixture only after reduction of the hexacyanoferrate (III), when the HPAP had been already hydrated. We now show that some contradictions exist between the data on the aldolase–GAPDH interaction and point out that the observations made by Patthy & Vas (1978) can be also explained without postulating substate channeling.

Apart from the numerical difference between the above K_d value and that of 3×10^{-7} M derived by Ovádi *et al.* (1978) for the same bienzyme complex, a further contradiction arose from the finding that syncatalytic inactivation of GAPDH by aldolase did not require preincubation, but it was observed practically immediatedly after mixing the two enzymes. Thus, the phenomenon could not be consequence of a slow complex formation, as suggested on the basis of fluorescence polarization measurements using fluorophores attached either to aldolase or GAPDH (Ovádi *et al.*, 1978). In addition to the above disagreements, during the past 10 years experimental data have been accumulated that argue against the concept of intermediate channeling in glycolysis (e.g. Masters & Winzor, 1981; Ehrlich, 1987; Kvassman *et al.*, 1988; Chock & Gutfreund, 1988; Kvassman & Pettersson, 1989; Vas & Batke, 1990; Wu *et al.*, 1991; Rognstad, 1991; Brooks & Storey, 1991). Many of the controversial data have been reviewed and discussed (Friedrich, 1985; Keleti *et al.*, 1989; Batke, 1989*a*,*b*, 1991; Ovádi, 1991) but, up to the present, consensus has not been reached in the literature.

In light of these contradictions we now reinterpret the experimental data on the aldolase-catalysed inactivation of GAPDH. These data are those of Patthy & Vas (1978) as well as our recent unpublished findings (1) that inactivation of GAPDH is due to modification of its active site thiol by HPAP and (2) that 3-phosphoglycerate kinase, although inactivated by nascent HPAP, will not protect GAPDH, whereas glycerol-3-phosphate dehydrogenase, although not inactivated by HPAP, does protect GAPDH [experiments were carried out with 3-phosphoglycerate kinase or glycerol-3-phosphate dehydrogenase as additional constituents in the system of Patthy & Vas (1978)]. These data corroborate, at face value, the previous assumption about the direct metabolite transfer; however, as outlined below, an alternative explanation can be provided.

Active-site directed inhibitors produced by the target enzyme (here aldolase) may react also with amino acid side chains of another protein molecule (here GAPDH) only if these residues are brought within the action radius of the *in situ* reagent. It is known that only the nascent dioxo-form of HPAP is reactive and that its hydration is much faster than its reaction either with arginine (Patthy, 1978) or with thiol (Lienhard & Jencks, 1966; Vander Jagt *et al.*, 1975). Thus, if HPAP is released from the aldolase active site into the aqueous solution its hydration occurs with much higher probability than its reaction. On this basis it was assumed (Patthy & Vas, 1978) that HPAP can react with GAPDH only at the site of its synthesis, which would be only possible within the molecule of an aldolase–GAPDH bienzyme complex.

We will now investigate more rigorously what the radius of action of the reagent produced *in situ* may be, i.e. what distance can be covered by nascent HPAP without significant hydration. This requires that, in addition to the relative rates of hydration and chemical reaction, discussed above, the relative rates of diffusion and hydration should be taken into account. According to Rashevsky (1960) the transient time of random diffusion ($\tau_{diff.}$) is given by the formula of a^2/D for a solute with a diffusion coefficient D moving into a linear dimension a. D can be taken as 5×10^{-6} cm^2/s which usually holds for small molecules in aqueous solutions (cf. e.g. Mastro *et al.*, 1984). In the present case a represents the distance between the protein molecules, which is obviously the function of protein concentration. Taking a value of 50 nm^3 as an approximate volume of a single spherical protein molecule with a size of about 150 kDa [its diameter was taken to be 7 nm (cf. Friedrich, 1984)], and at the simplest case assuming a uniform distribution of the molecules of this size in a cubic close-packed lattice, the inter-surface distance between the neighbouring molecules (i.e. a) was calculated according to the method published as an appendix by Endre & Kuchel (1986). The transient times of HPAP diffusion within this distance were also calculated using the formula given above at the different enzyme concentrations used mostly in the experiments (Table 1).

These values should then be compared to the transient time of

This paper is dedicated to the memory of Professor Tamás Keleti (deceased 4 October 1989), whose inspiration and initial encouragement made us think along these lines.

Abbreviations used: GAPDH glyceraldehyde-3-phosphate dehydrogenase; HPAP, hydroxypyruvaldehyde phosphate.

Table 1. Calculated distances between neighbouring globular protein molecules (150 kDa, diameter 7 nm) of different concentrations and the respective diffusion transient time of HPAP (see the text)

Protein concentration (M)	Distance between molecules (nm)	Transient time of diffusion, $\tau_{diff.}$ (μs)
10^{-6}	126	31.8
10^{-5}	54.7	6.0
10^{-4}	21.7	0.94
10^{-3}	6.3	0.08

HPAP hydration. In the literature the apparent first order rate constants of hydration of oxo compounds varies within a relatively wide range of about 0.1–10 s^{-1} (Jencks, 1964; Creighton *et al.*, 1988; Rae *et al.*, 1990), which would correspond to a value of transient time of about 0.1–10 s. Although precise data about HPAP hydration could not be found in the literature, as it is a very reactive dioxo compound, one can assume a hydration rate constant several magnitudes larger, but still far below the value of a diffusion-limited process. For example, if the hydration rate constant of 10^5 s^{-1} is assumed (which value is very probably an overestimation), this would mean a transient time of 10^{-5}s, i.e. $\tau_{hydr.} = 10$ μs would be estimated as a minimum value for HPAP hydration. One can see from Table 1 that the transient time of diffusion ($\tau_{diff.}$) is either comparable (at low protein concentrations) or shorter (at high protein concentrations) than this value.

Following the same line of argument, at sufficiently high protein concentrations (especially when GAPDH is in molar excess over aldolase), where the transient time of diffusion is extremely small, HPAP released from the aldolase active site can easily reach GAPDH molecules without significant hydration, simply by free diffusion, even if GAPDH is not complexed with aldolase. Then HPAP can bind to GAPDH, possibly through electrostatic interactions (GAPDH possesses a specific anion-binding site in its centre; cf. Harris & Waters, 1976; Leslie & Wonacott, 1984), followed by chemical reaction, the rate of which is largely enhanced by the high local concentration of the bound reagent. Thus, chemical modification of GAPDH with the nascent reagent may occur even if it is not complexed with aldolase. For the same reasons, at constant aldolase concentration, when GAPDH concentration is high enough, the efficiency of GAPDH inactivation is maximal and does not depend on its concentration. At lower protein concentrations, however, the transient times of hydration and diffusion are rather comparable and therefore at this range, e.g. at the same aldolase and much lower GAPDH concentrations, the inactivation efficiency is proportional to the concentration of GAPDH.

Thus, the relative transient times of diffusion and hydration can change depending on the protein concentrations and can result in a saturation-type dependence of the inactivation efficiency on the concentration of GAPDH, as was actually observed by Patthy & Vas (1978). The same relation, i.e. a higher extent of HPAP hydration, can be the reason for the finding of much smaller extent of GAPDH inactivation when GAPDH was added only later to the aldolase suicide mixture. Furthermore, the specific protective effect of glycerol-3-phosphate dehydrogenase (and no effect by 3-phosphoglycerate kinase) against the aldolase-catalysed inactivation of GAPDH can also be equally attributed either to the specificity of enzyme–enzyme interactions [glycerol-3-phosphate dehydrogenase has been shown to form bienzyme complexes both with aldolase (Batke *et al.*, 1980; Ovádi *et al.*, 1983, 1985; Vértessy & Ovádi, 1987; Vértessy *et al.*, 1991) and GAPDH (K. B. Nazaryan & J. Batke, personal communication)] or to differences in amino acid side chains modified and/or rates of modification in cases of different enzymes.

Alternatively, if the hydration rate constant of HPAP is not so high, but comparable to the above values characteristic for other oxo-compounds, $\tau_{hydr.}$ would be higher than $\tau_{diff.}$ in all protein concentrations investigated. In this case the explanation of the experimentally found saturation-type dependence of the inactivation efficiency of GAPDH on its concentration is not so plausible as described above. However, the efficiency is certainly affected by the molar ratio of aldolase and GAPDH as well as the relative reactivities of their side chains, which might equally result in such a dependence of the inactivation efficiency on GAPDH concentration within the investigated range and might account for this previous observation.

If either of the arguments hold, it is not necessary to assume a channeling of HPAP between the active sites of aldolase and GAPDH to account for the observed inactivation of GAPDH, as did Patthy & Vas (1978). It should be also noted that statistical collisions between protein molecules may further increase the transfer possibility. In fact, a hypothetical model for direct transfer of metabolites through productive collisions of enzyme molecules has been put forward by Friedrich (1984).

At present no suitable methodology is available for us to distinguish between the reagent-channeling and free-diffusion models for the aldolase-catalysed inactivation of GAPDH. Here we only point out that both the data of Patthy & Vas (1978) and our further unpublished observations can be equally explained by assuming either channeling of the aldolase-produced reagent to the target GAPDH through enzyme–enzyme interactions or free reagent diffusion between the active sites of noninteracting enzymes.

Our considerations also have general relevance in respect of kinetic investigations of metabolic transfer between enzyme active sites, especially in case of the so-called trapping methods (e.g. Ovádi & Keleti, 1978; Friedrich *et al.*, 1977; Solti & Friedrich, 1979; Datta *et al.*, 1985). Often the lack of an otherwise fast side-reaction (trapping) of a certain metabolite relative to its transformation by the next enzyme in the metabolic route is taken as indirect but unequivocal evidence in favor of channeling. However, the intermediate metabolite may often be able to reach the active site of the next enzyme by free diffusion even before its trapping by a side reaction (e.g. hydration, decomposition or reaction with an enzyme probe). If it is so, the possibility of the side (trapping) reaction can be reduced and the metabolically required enzymic transformation is facilitated by the fast formation of specific enzyme-substrate interactions (e.g. covalent adduct formation, etc.), or simply by increasing the local concentration of the intermediate at the enzyme active site.

Furthermore, for the same reasons and considering that the *in vitro* and *in vivo* diffusion rates of small molecules as metabolites are comparable (Jacobson & Wojcieszyn, 1984) and that *in vivo* protein concentrations are relatively high (Srere, 1967), we argue that, in general, enzymic transformation of an unstable intermediate (whose decomposition rate might be much faster than the rate of enzymic transformation) through its usual metabolic pathway does not necessarily require channeling.

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Lysosomal handling of cystine residues: stoichiometry of cysteine involvement

In a earlier contribution to this journal (Lloyd, 1986) I addressed the question of whether cystine residues in proteins are reduced in the course of proteolytic degradation in lysosomes. The available evidence appeared contradictory. On the one hand, several investigators had shown that the presence of thiol is necessary for the efficient degradation of disulphide-containing proteins by lysosomal enzymes and that this requirement could not be attributed solely to the thiol-requirement of some of the proteinases. On the other hand, cystine accumulates in the lysosomes of cells of cystinosis patients, in which the lysosome membrane cystine porter is absent, and this accumulation correlates with the degradation of proteins that contain cystine residues.

I proposed that the apparent conflict resolves if disulphide-containing proteins are indeed reduced during lysosomal proteoloysis and if cysteine is the reducing agent. If this is the case, the cysteine that is generated in the process, and which accumulates in cystinosis, arises from the reducing agent.

Subsequently a report (Pisoni *et al.*, 1990) appeared describing the properties of a human fibroblast lysosome membrane porter specific for cysteine and its close analogue cysteamine. The pH-profile of its activity is unusual: it transports cysteine poorly below pH 6, and shows maximal activity at around pH 7.4. This observation was taken by Pisoni *et al.* (1990) as indicating that the porter's role is to transport cysteine into the lysosome. By contrast, most of the other lysosome membrane porters are assumed to mediate the efflux of metabolites from the lysosome. Pisoni *et al.* (1990) presented their observations as supporting evidence for the role I had earlier proposed for cysteine in the lysosomal breakdown of disulphide-containing proteins.

Recently two reports have appeared in which it is proposed that the reduction of disulphide bridges in lysosomes is a crucial step in protein processing by antigen-processing cells. Collins *et al.* (1991) have synthesized a conjugate in which [^{125}I]iodotyrosine is linked to proteins through a disulphide moiety. [^{125}I]Iodotyrosine is liberated when these conjugates are endocytosed and taken to the lysosomes, indicating disulphide reduction in this subcellular compartment. Jensen (1991) has shown that lysosomal reduction of disulphide bridges in the insulin molecule is a key component in A-chain recognition by class II-restricted T lymphocytes.

I am concerned that in these subsequent publications the stoichiometry of the mechanism I proposed for intralysosomal disulphide reduction has not been fully appreciated. Consider the digestion of a protein that contains cystine but no cysteine moieties. If, as I proposed (Lloyd, 1986), the reducing agent is free cysteine, the cysteine consumed will be exactly balanced by the cysteine liberated when the disulphide moieties in the protein are reduced and its peptide linkages hydrolysed by the cathepsins. There will also be an equivalence between the cystine content of the proteins entering the lysosomes by endocytosis or autophagy and the cystine released from the lysosomes via the cystine porter. It follows that there is no requirement for a net influx of cysteine from the cytoplasm across the lysosome membrane. Pisoni *et al.* (1990) postulate that the lysosome membrane cysteine porter constitutes a major route for the delivery of cysteine to the lysosomal compartment. While this may be potentially so, I question the existence of a significant flow of cysteine into lysosomes under physiological conditions. If there is an inward flow, it is likely to constitute an anaplerotic pathway, serving only to maintain the cysteine concentration within the lysosomes by replacing any cysteine that undergoes autoxidation to cystine.

Many cystine-containing proteins also have cysteine residues. When such proteins are degraded in the lysosomes, the consequence will be a net production of both cysteine and cystine. Even allowing for some loss of cysteine by autoxidation, a net efflux of both amino acids across the membrane is likely. Whether cysteine is transported out on the cysteine-specific porter, despite