1. BINDING OF NADH TO LDH[1]

Reduced nicotinamide adenine dinucleotide (NADH) fluoresces at 460 nm when it is excited at 340 nm, and this fluorescence has been found to be enhanced in the presence of lactate dehydrogenase (LDH). This effect, which is believed to be due to a change in the environment of the coenzyme when it binds LDH, was used to determine the stoichiometry of binding. To 2 ml of a 0.473 mg/ml solution of beef heart LDH in 0.05 M Tris-acetate buffer, pH 7.2, were added small amounts of a 1 mM NADH solution in the same buffer. The fluorescence emission at 460 nm was measured (in arbitrary units) after each addition and, as a control, readings were also made of the fluorescence changes when NADH was added to 2 ml of the same buffer *minus* enzyme. The results are shown in Table 1.

Table 1:

Total volume of NADH added (µl)	Fluorescence when added to LDH (units)	Fluorescence when added to buffer (units)
0	0	0
5	1.3	0.3
10	2.5	0.6
15	3.9	0.9
20	5.1	1.2
25	6.1	1.6
30	6.8	1.9
35	7.2	2.2
40	7.5	2.5
50	8.1	3.1
60	8.8	3.8
70	9.4	4.4
80	10.0	5.0

The molecular weight of LDH was estimated by gel-filtration on a 50 x 2.5 cm column packed with Sephadex G-200. The column was equilibrated with 0.05 M Tris-acetate buffer, pH 7.2, and approximately 1 mg samples of a number of proteins, whose molecular weights were known, were applied successively to the column and eluted with buffer. The concentrations of protein in the effluent were estimated by measurements of the absorbance at 280 nm, and the volume of buffer necessary to elute each protein from the column was determined. The results are shown in Table 2 together with the elution volume of LDH when it was chromatographed under the same conditions.

Table 2:

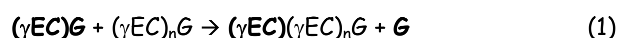
Protein	Molecular weight (x 10^{-3})	Elution volume (ml)
Serum albumin (monomer)	65-70	147
Serum albumin (dimer)	130-140	127
Phosphatase (from *E. coli*)	75-80	145
Aldolase (from rabbit)	140-150	127
Ceruloplasmin (human)	150-165	124
R-Phycoerythrin	250-270	110
α-Conarachin	285-305	107
Apoferritin	460-490	93.5
β-Galactosidase	510-530	91
LDH	-	125

On the basis of these data, what do you infer concerning the stoichiometry of binding of NADH to LDH and the likely overall subunit composition of the enzyme?

Note that at the relatively high LDH protein concentrations employed in these studies, dissociation of the enzyme-NADH complex is so slight that binding can be regarded as being essentially irreversible.

2. DIPEPTIDYL OR TRIPEPTIDYL TRANSPEPTIDASE?

Phytochelatins (PCs), (γ-Glu-Cys)$_n$-Xaa polymers derived from glutathione (GSH, γ-Glu-Cys-Gly) and related thiol peptides by the action of the enzyme PC synthase play a pivotal role in heavy metal tolerance in plants, some fungi and possibly some animals by chelating toxic metal ions, such as Cd^{2+}, and decreasing their free concentrations. In most studies of partially purified preparations of the enzyme, PC synthase-catalyzed PC synthesis from GSH has been considered to proceed by the transpeptidation of a γ-Glu-Cys (γEC) unit from one GSH molecule to another to form PC_2 (= (γEC)$_2$G) and, after the accumulation of sufficient (substrate) levels of PCs, by the transpeptidation of a γEC unit from GSH or a PC to another PC (PC_n) molecule to form PC_{n+1}. Appropriately, PC synthase has been defined as a γ-Glu-Cys dipeptidyl transpeptidase and thought to catalyze a reaction of the type:



$$(\gamma EC)G + (\gamma EC)_nG \rightarrow (\gamma EC)(\gamma EC)_nG + G \qquad (1)$$

Implicit, therefore, has been the assumption that PC chain extension proceeds C to N with cleavage of the Cys-Gly peptide bond of the donor, not the acceptor. Inspection of the literature – a literature based exclusively on the partial characterization of impure enzyme preparations – however, reveals that the notion that PC synthase is a tripeptidyl transferase is equally tenable. There are no published data to refute a scheme in which PC synthase catalyzes the transfer of γ-Glu-Cys-Gly units in a reaction of the type:

$$(\gamma EC)G + (\gamma EC)_nG \rightarrow (\gamma EC)_n(\gamma EC)G + G \qquad (2)$$

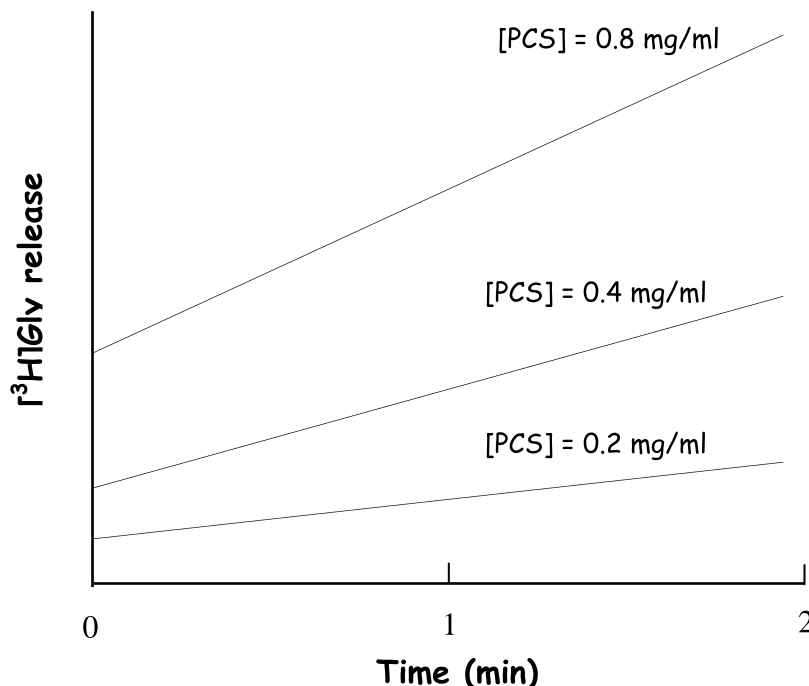
If this were the case, PC chain extension would proceed N to C, not C to N, after cleavage of the Cys-Gly peptide bond of the acceptor, not the donor.

Imagine that you have recently cloned a cDNA encoding the enzyme PC synthase (PCS) and have a ready supply of pure catalytically active recombinant enzyme. The enzyme comprises a single polypeptide species of M_r 55,000 which alone is sufficient for catalysis. Assume that you have ample supplies of [^{3}H-Gly]GSH, [^{35}S-Cys]GSH and unlabeled GSH, and can make and purify as much unlabeled or labeled PC_2 and/or PC_3 as you need for your experiments.

A. Suggest a series of relatively simple experiments using these reagents that would enable you to determine if PC synthase is a dipeptidyl or tripeptidyl transpeptidase. Explain where complications might arise especially in experiments requiring longer incubations of the enzyme with its substrates.

B. An observation that has perplexed researchers for a number of years is that whenever long-chain PCs are found in cells, they are accompanied by *des*(Gly)PCs, (γ-Glu-Cys)$_n$ polymers lacking a C-terminal residue. Which of the two schemes, dipeptidyl transfer or tripeptidyl transfer, most readily accounts for the synthesis of *des*(Gly)PCs? Explain.

C. When high concentrations of PCS are added to media containing [^{3}H-Gly]GSH and the appearance of free [^{3}H]Gly in the incubation medium is measured, the results shown in the figure below are obtained. Note the non-zero intercepts on the ordinate, which you suspect represent extremely rapid [^{3}H]Gly release (release at a rate exceeding the time-resolution of your measurements). How do you account for the kinetics of [^{3}H]Gly release? Propose experiments to further test your proposal.



D. A property of PC synthase that you initially find confusing is that the substitution of any one of its 41 Ser residues to Ala or Cys by site-directed mutagenesis has little or no effect on catalytic activity. Disappointed by the negative outcome of these experiments, you decide to do the inverse experiment – to singly mutate each of the 15 Cys residues to Ser or Ala residues. In so doing you find that substitution of Cys^{91} to Ser only partially abolishes activity while an Ala substitution at the same position completely abolishes activity. Substitution of any one of the other 14 Cys residues with Ser or Ala has little or no effect on activity. On the basis of these results and the time-dependence of free Gly release shown above, propose a model for the role(s) played by Cys^{91} in catalysis which is capable of explaining the partial activity of C91S mutants and complete lack of activity of C91A mutants. Note that while C91S mutated enzyme elicits a seemingly instantaneous release of free [^{3}H]Gly from [^{3}H-Gly]GSH, C91A mutated enzyme does not. Neither C91S nor C91A mutated enzyme mediate the slower steady rate of [^{3}H]Gly release seen after the initial burst.

3. MALARIAL MEMBRANE-ASSOCIATED PPASE

You isolate membrane vesicles from the malarial parasite *Plasmodium falciparum* and discover that the addition of inorganic pyrophosphate (PPi), Mg^{2+} and KCl elicits high rates and extents of intravesicular acidification. Further you note that agents such as gramicidin-D that abolish PPi-dependent intravesicular acidification increase the rate at which the vesicles hydrolyze PPi to Pi. Excited by the possibility that you may have stumbled into a new target for the action of antimalarial drugs (globally, malaria is still one of the most widespread and debilitating diseases) you decide to investigate this activity further.

A. What type of activity do you appear to have discovered? How do you explain the capacity of agents such as gramicidin-D to increase the rate of PPi hydrolysis? What other agents would you expect to have a similar effect? Explain.

B. Both PPi hydrolysis and PPi-dependent intravesicular acidification are obligatorily dependent on the provision of millimolar K^+. The requirement for K^+ for PPi hydrolysis is seen even in media containing gramicidin-D. How do you explain these results? Is

K^+ exerting its effects *via* the inside-acid H^+ gradient established by PPi hydrolysis or is it acting on the enzyme directly? Explain.

C. We are all accustomed to thinking of ATP as being the principle “energy currency” in living cells in that this compound is generated by both anaerobic and aerobic metabolism. The presence of a PPi-dependent H^+ pump in *Plasmodium* membranes suggests that not only ATP but also PPi can act as an energy source for some processes in some organisms. What types of metabolic reactions might be responsible for the net formation of PPi in *Plasmodium* (or its mammalian host)?

D. An intriguing feature of the PPi-dependent H^+ pump from *Plasmodium* is its exquisite sensitivity to inhibition by vanadate. Inclusion of micromolar concentrations of vanadate in the assay medium completely abolishes PPi hydrolysis and intravesicular acidification. What might this signify concerning the catalytic mechanism of this enzyme? How would you test your proposal? Is vanadate a good candidate platform compound for the development of antimalarial drugs – drugs that are unlikely to have adverse effects on the mammalian host? Explain.

E. As a prelude to studies directed at the crystallization of the *Plasmodium* PPi hydrolase for drug design purposes, you attempt to purify the enzyme. Table 3 below summarizes the results of your preliminary attempts at purification. *Plasmodium* membranes were prepared by density gradient centrifugation, solubilized with the nonionic detergent Triton X-100, and the 200,000 g supernatant from the solubilizations was subjected to gel-filtration on a column packed with Sephacryl S-400. All the Sephacryl S-400 fractions containing PPi hydrolytic activity (which coeluted in a single peak) were then subjected to anion-exchange chromatography on a column packed with Mono-Q. Fill in the gaps (??s) in the purification table.

Table 3:

Step	Specific activity* (µmol/mg/min)	Purification (fold)	Recovery (step) (%)
Plasmodium membranes	0.22	1.0	100.0
<u>*Gel-filtration*</u>			
Total fractions	-	-	96.5
Peak activity	1.47	??	-
<u>*Anion-exchange*</u>			
Total fractions	-	-	42.5
Peak activity	18.33	??	-
Overall	-	??	41.0

*1 µmol/mg/min = 1 µmol PPi hydrolyzed to 2Pi per mg protein per min

F. Assume that SDS-PAGE of the final peak activity chromatographic fractions from the Mono-Q column (those possessing a specific activity of 18.33 µmol/mg/min) reveals a single intense protein band at M_r 68,000. Does this enzyme appear to be abundant in the starting material? Explain your reasoning. [A membrane protein is said to be relatively abundant when it represents 0.5% (w/w) or more of the protein in a given membrane fraction].

G. A valid concern you have is that there may be other PPi hydrolytic activities in the starting material. From what you know of the properties of the *Plasmodium* enzyme,

how might you design your PPi hydrolytic assays to maximize the likelihood that the PPi hydrolytic activity you measure is primarily attributable to the enzyme responsible for intravesicular acidification and not other PPi hydrolases? Explain.

H. A common problem associated with the ascription of specific polypeptides to enzymes on the basis of SDS-PAGE analyses of purified preparations is that the dominant polypeptides(s) on SDS-gels is(are) not necessarily the one(s) of interest. For instance, if the enzyme responsible for PPi-dependent intravesicular acidification in *Plasmodium* membranes possesses a very high turnover number, the amount of enzyme-specific polypeptide needed to measure activity may be insufficient to give a clear protein band on an SDS-gel. Again, from what you know of the PPi-dependent H^+ pump from *Plasmodium*, how would you determine if the M_r 68,000 band on SDS-gels of the partially purified enzyme is or is not a subunit of the enzyme? How might you use a similar approach to obtain an estimate of the abundance of this enzyme in the starting material (*Plasmodium* membranes)? Explain your reasoning and any difficulties, interpretative or experimental, that might arise.

I. It is generally assumed that the free energy of hydrolysis of ATP to ADP + Pi is greater than the free energy of hydrolysis of PPi to Pi. Is this necessarily true? If the standard free energy of hydrolysis of PPi to Pi is –33.5 kJ/mol and the prevailing Pi concentration in *Plasmodium* is 1 mM, what is the minimum concentration PPi would have to achieve for its free energy of hydrolysis to be equivalent to that of ATP? Is such a mass action ratio compatible with the K_m of the *Plasmodium* enzyme for PPi (5 μM)? If extravesicular pH is 7.0, what is the minimum intravesicular pH that the *Plasmodium* enzyme could achieve under these conditions if $1H^+$ is delivered into the interior of the vesicles for every PPi hydrolyzed? Assume that electroneutrality is maintained across the membranes of the vesicles by the rapid equilibration of Cl^- and that intravesicular buffering capacity is negligible.

[1]Modified from *Biochemical Reasoning* (1972), Kerridge, D. and Tipton, K.F., eds., W.A. Benjamin, Inc.