Is pyridoxal 5'-phosphate an affinity label for phosphate-binding sites in proteins?: the case of bovine glutamate dehydrogenase

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The effects of pyridoxal 5'-phosphate (PalP) on ox liver glutamate dehydrogenase (94% inactivation by 1.8 mM reagent at pH 7 and 25 °C) have been compared with those of three analogues, 5'-deoxypyridoxal (96% inactivation), pyridoxal 5'-sulphate (97%) and pyridoxal 5-methylsulphonate (94%), in order to establish whether PalP acts as an affinity label for this enzyme. Like PalP and unlike pyridoxal, which is a much less potent inactivator, none of the analogues has a free 5'-OH group to cyclize with the aldehyde function. The result with 5'-deoxypyridoxal shows that a negative charge, such as that of the phosphate group, is not required for efficient inactivation. With all four reagents, addition of an excess of cysteine or lysine led to 90–100% re-activation over 3–20 h. Dialysis also caused re-

activation to a similar extent. A combination of 2.15 mM NADH, 1 mM GTP and 10 mM 2-oxoglutarate gave complete protection against PalP, but only partial protection against the analogues. 5'-Deoxypyridoxal still caused 20–25% inactivation in the presence of the protection mixture. Absorbance measurements after reduction with NaBH₄ show the characteristic features of a reduced Schiff's base and allowed estimation of the extent of reaction. With all the reagents the protection mixture decreased incorporation by about 1 mol/mol, but levels of incorporation without protection varied from about 2 mol/mol for PalP up to about 5 mol/mol for 5'-deoxypyridoxal. The labelling at additional sites may explain the residual inactivation in the presence of potent protecting agents.

INTRODUCTION

Pyridoxal 5'-phosphate (PalP) serves as a prosthetic group for various enzymes and is normally attached by Schiff's-base linkage to a protein lysine residue [1]. The same compound also reacts with many other proteins, however, and since the reaction in the absence of light tends to be fairly specific for lysine residues, PalP has become very popular as a versatile reagent for protein modification (see, e.g., [2–11]). The covalent reaction may be reversed by dialysis or dilution (see, e.g., [2–4,7]) or by addition of an excess of an aminothiol [5,8] or amine [2,3], or alternatively labelling may be made more permanent by reduction with borohydride [1–11], in which case the built-in chromophore offers a convenient means of approximate estimation of the extent of labelling [12] and the possibility of identifying modified peptides [9–11]. Even when the label has been thus stabilized by reduction of the imine to an amine, it may, if necessary, be removed by strong illumination [13] to regenerate the original unmodified lysine residue. The chromophore also allows the label to be used as a probe. Thus, in glutamine synthetase, perturbations of the absorption spectrum of the label were used to monitor binding of metal ions, allosteric inhibitors and substrate [14], and, in aspartate transcarbamoylase, fluorescence measurements of the bound chromophore were used to measure distances between catalytic sites [15]. Finally, it appears that, in many cases of protein modification by PalP, the reaction involves a relatively slowly adjusting equilibrium between covalently and non-covalently bound reagent [16]. If the modification leads to loss of biological function, different levels of partial inactivation may be measured with great precision [17,18]. This opens up the possibility of 'equilibrium protection studies' [19], a convenient means of studying partial or complete protection by a variety of ligands. This approach, developed for bovine glutamate dehydro-

genase (GDH), has now been applied to many other systems (see, e.g., [20–22]).

Given that PalP is selective for lysine residues, the reagent has also been found to be rather specific in two further senses: firstly, it frequently modifies only one or two lysine residues in a protein [4,8–11,23], although most proteins have many lysine residues and these tend to be on the surface; secondly PalP may modify where pyridoxal, lacking the phosphate group, either does not (see, e.g., [3,4,11]) or else does so much less effectively [2,5,6,24]. Specificity of the first kind may be explained in terms of the unique environment of an individual lysine residue. It has been suggested that the reactive lysine residues in ribonuclease A [11] and in bovine GDH [25] are more susceptible to attack by virtue of abnormally low pK values, perhaps reflecting a hydrophobic environment (but see [26] for discussion of the pH-dependence of Schiff-base formation). Such factors alone, however, cannot explain specificity of the second kind; indeed such an environment might be thought to favour reaction by the uncharged pyridoxal. There is accordingly a widespread view that PalP acts essentially as an affinity labelling reagent, steered into the active sites of enzymes with phosphorylated substrates by its own phosphate group (see, e.g., [3,6,7,9,11,27]). On the other hand, Anderson et al. [2], discussing the relative reactivities of pyridoxal and PalP with bovine GDH, offer an alternative explanation. Pyridoxal exists largely as an internal hemiacetal, which cannot be formed in PalP, since the 5'-OH group is blocked [28,29]. Thus a higher effective aldehyde concentration may account for the apparently enhanced reactivity of PalP [28–30].

The availability of a range of PalP analogues differing in their substitution at the 5'-position [31–33] offers the possibility of testing directly whether the phosphate group is essential for efficient labelling. In the present paper we have investigated the

Abbreviations used: PalP, pyridoxal 5'-phosphate; GDH, glutamate dehydrogenase.

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Figure 1 Molecular structure of PalP and the analogues used in the present study

The structure shown represents one of the possible tautomeric forms present in a pH-dependent equilibrium in solution. The four compounds compared in the present study differ in their substitution (X) at the 5-position of the pyridinium ring. In PalP it is $-\text{CH}_2\text{OPO}_3^{2-}$; in pyridoxal 5'-sulphate X is $-\text{CH}_2\text{OSO}_3^-$; in pyridoxal 5-methylsulphonate, X is CH_2SO_3^- ; in 5'-deoxypyridoxal, X is CH_3 .

reaction of bovine GDH with several such compounds. These include the 5'-sulphate [32] and 5-methylsulphonate [33] analogues, in which phosphate is replaced by negatively charged groups of different size, and 5'-deoxypyridoxal [31], which has no negative substituent, but still cannot cyclize, since there is no 5'-OH group (Figure 1).

MATERIALS AND METHODS

Bovine GDH (EC 1.4.1.3), obtained from Biozyme Laboratories (Blaenavon, Gwent, Wales, U.K.) as a suspension in $(\text{NH}_4)_2\text{SO}_4$ solution, was prepared for use as described elsewhere [34]. Nucleotide cofactors were purchased from Boehringer Corp. (London), Lewes, Sussex, U.K. Pyridoxal 5'-phosphate was from Koch-Light Laboratories (now part of Genzyme Ltd.), and its purity was confirmed by t.l.c. 5'-Deoxypyridoxal was synthesized as described by Iwata [31] and purified by vacuum sublimation. Pyridoxal 5'-sulphate was prepared by the method of Kuroda [35], as adapted by Yang et al. [32]. In the synthesis of pyridoxal 5-methylsulphonate [33], 5'-bromo-5-deoxypyridoxamine was first prepared as described elsewhere [36]; 1 g of this compound was dissolved in 5 ml of water and slowly added, with stirring, to a solution containing 0.7 g of Na_2SO_3 in 5–10 ml. After 24 h, the solution was concentrated to about 2 ml, and 0.5 ml of 6 M HCl was added. The product precipitated within a few minutes at room temperature and was recrystallized from 1 M HCl and further purified on Dowex 50W-X8 cation-exchange resin.

Dialysed enzyme at either 0.2 mg or 2 mg/ml was incubated at 25 °C with PalP or one of the three analogues at a concentration of 1.8 mM unless otherwise indicated. Samples were protected from light with metal foil. At timed intervals, portions (2–20 μl) were withdrawn with a constriction micropipette for fluorimetric assay [16,17,37] of enzyme activity in a 4 ml reaction mixture containing 40 mM L-glutamate and 240 μM NAD^+ in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C. Whenever possible a small sample volume (2 μl) was used in order to minimize 'artifactual' blanks due to the transfer of PalP or its analogues. At less frequent intervals measured samples (1–2 mg of GDH) were taken and a portion of each sample was treated with freshly prepared ice-cold NaBH_4 , which discharged the yellow colour of PalP. After 1 h at 4 °C these samples were dialysed against four

changes of 0.1 M potassium phosphate buffer, pH 7.0, at 4 °C and clarified by centrifugation. The remainder of each sample was dialysed without exposure to NaBH_4 . After dialysis both the reduced and the unreduced samples were used to record absorption spectra from 220 to 400 nm (Cary 219 scanning spectrophotometer; Varian Instruments, Palo Alto, CA, U.S.A.), to determine protein concentration from static readings of A_{280} and to assay for residual enzyme activity as described above. For the spectrophotometric measurements the enzyme samples were diluted into guanidinium chloride (6 M final concn.) in 0.1 M phosphate buffer, pH 7, in order to eliminate possible local effects in the folded protein that might lead to variability in the absorption coefficient for the bound label. The peak-to-peak absorbance ratio A_{325}/A_{280} was used as an indicator of the stoichiometry of incorporation, with a value of 0.21 being taken to indicate 1 mol of PalP or analogue bound/mol GDH subunit [16,17].

The concentration of NaBH_4 necessary to achieve quantitative immobilization of the label was investigated with some care after indications that a protocol relying merely on decolorization (reflecting reduction of excess PalP to pyridoxol 5'-phosphate) gave variable results. Under our conditions, additions of less than 10 mM NaBH_4 gave only 70–90% of the maximal incorporation, and 40 mM NaBH_4 was therefore routinely used. This gave enzyme activities after reduction and dialysis that were in excellent agreement with those measured on samples taken straight from the inactivation incubation without reduction.

RESULTS AND DISCUSSION

Figures 2(a)–2(d) show the results of incubating GDH with PalP or its analogues. The result in Figure 2(a) shows a familiar pattern [2,16,25,38] in which activity declines over about 20 min to a residual value, in this case 6% of the starting activity, which then remains constant for several hours. This is known to be due to the modification of Lys-126 [10]. In the case of GDH from *Clostridium symbiosum*, the corresponding residue is also modifiable with PalP [39] and has lately been shown through crystallographic studies [40] to be directly involved in catalysis rather than in substrate binding. This is in agreement with the inference from protection studies of the bovine enzyme [17].

The apparent paradox of residual activity, albeit low, after the chemical modification of a catalytically essential residue [38] was resolved by the demonstration [16], as mentioned above, that the covalently modified enzyme is in equilibrium with a non-covalent GDH–PalP complex.

Figures 2(b)–2(d) show clearly that all three of the analogues employed inactivate bovine GDH in a strikingly similar way. In all cases reaction appears effectively to have reached equilibrium within 20 min. The level of residual activity achieved with pyridoxal 5'-sulphate (3%) and 5'-deoxypyridoxal (4%) is even lower than with PalP, whereas pyridoxal 5-methylsulphonate gives the same (6%) final activity as PalP. None of these analogues has a 5'-OH group, and hence, like PalP, they should all deliver the full complement of aldehyde. Unlike the sulphate and sulphonate analogues, however, 5'-deoxypyridoxal has no negative charge to mimic that of the phosphate in PalP, and yet it is clearly an effective inactivating agent. This single observation appears to call into question any view that PalP behaves as an affinity label for GDH, steered into the phosphate-binding sites for the coenzyme.

The reversibility of the inactivation reactions was tested in three ways. At various times (20, 60 and 120 min), after the final level of activity had been attained, samples were withdrawn from each incubation mixture and treated with a large excess of either

Figure 2 Re-activation of GDH by treatment with lysine or cysteine following treatment with PaIP and its analogues

GDH (2 mg/ml) was incubated as described in the Materials and methods section with PaIP (a), pyridoxal 5'-sulphonate (b), pyridoxal 5-methylsulphonate (c) or 5'-deoxypyridoxal (d). In all cases the concentration of modification reagent was 1.8 mM. At intervals over 20 h (note break in horizontal axis) 2 μ l samples were withdrawn for assay (○). From each incubation larger samples (0.1 ml) were removed at 20, 60 and 120 min and diluted 5-fold into either 180 mM L-lysine or 90 mM L-cysteine (final concns.) in 0.1 M phosphate buffer, pH 7.0. These diluted enzyme solutions were incubated at 25 °C; 10 μ l samples were withdrawn periodically from these re-activation incubation mixtures for activity assays. In each panel, ● indicates assays from the 20 min sample of the original incubation, ▲ denotes the 60 min sample, and ■ shows the 120 min sample. In all cases the continuous and broken lines indicate incubations with L-cysteine and L-lysine respectively. Where, as in most cases, the 20 h assay value is higher than the preceding one, a broken trace is shown, since there is not sufficient information to deduce the true time course.

Table 1 Recovery of GDH activity upon dialysis following inactivation by pyridoxal 5'-phosphate and various analogues

GDH was incubated with the four compounds indicated under the same conditions as for Figure 2. In each case assays of the progress of inactivation were carried out at 20, 60 and 120 min, and at these same time points a larger sample was withdrawn for dialysis at 4 °C. The paired rows compare activity before and after dialysis.

Reagent...	Residual activity (%)			
	Pyridoxal 5'-phosphate	Pyridoxal 5'-sulphate	Pyridoxal 5-methylsulphonate	5'-Deoxypyridoxal
After 20 min	6	4	7	3
After dialysis	108	100	100	100
After 60 min	6	5	5	4
After dialysis	98	92	100	108
After 120 min	5	4	5	4
After dialysis	102	92	100	94

L-lysine, pH 7 (final concn. after mixing with sample was 180 mM), or L-cysteine, pH 7 (final concn. was 90 mM), and incubated at 25 °C. Recovery of activity was monitored by the standard assay. Figures 2(a)–2(d) show that, in all four cases, there was ultimately a full recovery of activity, within experimental error. There were, however, significant differences in the pattern of recovery, depending upon which reagent was used to remove the chemical modifier. 5'-Deoxypyridoxal gave a very

similar pattern to PaIP, with 90 mM L-cysteine giving more rapid and complete recovery than 180 mM L-lysine, and approx. 50 % recovery within 1 h. With pyridoxal 5-methylsulphonate, L-cysteine was again the superior re-activating agent, although recovery was substantially slower, with only 20–30 % recovery in the first hour. Pyridoxal 5'-sulphate gave the most distinctive recovery pattern, with less than 10 % return of activity after 1 h with L-cysteine and slightly faster recovery with L-lysine initially.

The third re-activation procedure was dialysis, as described in the Materials and methods section. As shown in Table 1, regardless of the reagent used for inactivation, dialysis led to over 90 % recovery of activity, and samples taken at 120 min gave as good regain of activity as samples taken at 20 min. With all four reagents, therefore, the inactivation is reversible, with little sign of the secondary irreversible changes seen in the reaction of some other enzymes with PaIP (see, e.g., [41]).

Active-site and allosteric ligands give various extents of protection of GDH activity against the effects of PaIP [16–19]. In this study a combination was sought that would conveniently give full protection against PaIP so that the analogues could be tested under the same conditions. It had been reported that a mixture of 5 mM NADPH and 5 mM 2-oxoglutarate gave complete protection of activity [10], but in our hands 1.8 mM PaIP still gave about 50 % inactivation in the presence of these compounds, as judged by either the activity assay given above or the assay described in [10]. Finding that NADH gave better protection than NADPH, we explored various combinations of NADH (2.15 mM), GTP (1 mM) and 2-oxoglutarate (10 mM). NADH with GTP gave incomplete protection (about 30 %

Table 2 Incorporation of reagent by glutamate dehydrogenase treated with PalP or its analogues in the presence and absence of protecting agents

The enzyme was treated with the four reagents under the same conditions as in Figure 2. At the intervals indicated, samples (1 mg GDH) were withdrawn, treated with NaBH₄ as described in the Materials and methods section, thoroughly dialysed and clarified, and used for scanning spectrophotometry. With each reagent, paired incubations either included or excluded the combination of protection agents GTP (1 mM), NADH (2.15 mM) and 2-oxoglutarate (10 mM). Extents of incorporation were calculated from the ratio A_{325}/A_{280} on the assumption that a value of 0.21 represents incorporation of 1 mol of reagent bound per mol of GDH subunit [22,23].

Time (min)...	Incorporation (mol/mol of subunit)											
	PalP			Pyridoxal 5'-sulphate			Pyridoxal 5-methylsulphonate			5'-Deoxypyridoxal		
	20	60	120	20	60	120	20	60	120	20	60	120
Without protection	—	2.04	2.10	2.56	2.57	2.86	3.10	3.33	3.38	5.14	5.24	5.14
With protection	0.81	1.00	0.76	1.38	1.52	1.48	1.76	1.95	2.00	3.90	3.71	3.76
Difference	—	1.04	1.34	1.18	1.04	1.38	1.34	1.38	1.38	1.24	1.53	1.38

inactivation), but NADH and 2-oxoglutarate together, with or without GTP, protected completely. In comparative studies, the combination of NADH, GTP and 2-oxoglutarate, adopted as a standard protection mixture, also afforded very substantial protection against all the PalP analogues tested. With the analogues, however, the protection was incomplete. Average activity in all the incubations (conditions as in Figure 2, except for addition of protection agents) remained essentially constant between 30 min and 2 h at 100 % of the initial value (PalP), 90 % (pyridoxal 5'-sulphate), 88 % (pyridoxal 5-methylsulphonate) and 79 % (5'-deoxypyridoxal).

Purely on the basis of equilibrium considerations, the sample with pyridoxal 5'-sulphate should show the greatest resistance to protection, since this reagent gives the fullest level of inactivation and the slowest recovery upon addition of lysine or cysteine. However, the results indicate that, in the presence of the protecting agents, it is deoxypyridoxal that gives the greatest inactivation (20–25 % loss of activity). The possibility cannot be ruled out that this reflects a response to reaction at other sites on the protein. Accordingly the values for incorporation of reagent both with and without protection are of interest.

Absorption spectra were recorded after reduction with NaBH₄ and dialysis, and all showed the characteristic features [12] of a reduced Schiff's base. Table 2 shows the extent of incorporation of reagent into samples reduced and dialysed after 20, 60 and 120 min incubation with each of the reagents with and without protection. With all four inactivating agents the addition of protecting agents lowered the incorporation by about 1 mol/mol of subunit, consistent with the complete masking of Lys-126. Of the four reagents, PalP gives the lowest levels of overall incorporation, about 2 mol/mol without protection and 1 mol/mol with protection. The residual 1 mol/mol presumably largely reflects modification of Lys-333, which has been shown also to react with PalP [10]. The two other negatively charged reagents give slightly higher levels of labelling, rising to about 3 mol/mol with the 5-methylsulphonate, and the highest incorporation appears to be with 5'-deoxypyridoxal (about 5 mol/mol in the absence of protection). This may bear some relation to the residual inactivation noted above with this reagent, even in the presence of potent protecting agents.

In view of the experiments reported here, it is clear that, in the case of bovine GDH, PalP does not owe its efficacy as a modification agent to specific binding of the 5'-phosphate group at the active site. Other reagents lacking the phosphate group, and even lacking the negative charge entirely, still give potent inactivation. Since the same combination of protecting agents is

effective to various extents against all the reagents, it seems likely that the three analogues inactivate by reacting with the same residue as PalP, which cannot, therefore, be regarded as a true affinity label for this enzyme. It is nevertheless clear that the phosphate substituent does confer specificity in a negative sense, in that PalP fails to react as extensively as 5'-deoxypyridoxal with other lysine residues. This is true also for the sulphate and sulphonate analogues.

If one re-examines claims for affinity labelling of other enzymes by PalP, the case is convincingly made in a few cases. Rippa et al. [3] showed with 6-phosphogluconate dehydrogenase that four equivalents of pyridoxal could be attached to lysine residues without any loss of activity, and that the enzyme thus modified was still fully susceptible to subsequent inactivation by PalP, assumed therefore to be modifying other lysyl residues. Schnackerz and Noltmann [6] found that, in the case of phosphoglucose isomerase, 5'-deoxypyridoxal did inactivate to the extent of 70 %, but that this required a much higher concentration of reagent than required with PalP. In both cases it seems that the phosphate assists in delivering a reagent that would otherwise not reach the active site as easily, if at all. In general, however, the claim for affinity labelling by PalP is either purely speculative or else based on comparison with the effect of pyridoxal. As already pointed out many years ago [6], this is not the appropriate comparison to make, and, as our results emphasize, it can supply only equivocal evidence. If a case is to be made for affinity labelling of phosphate binding sites by PalP, a comparison with 5'-deoxypyridoxal would appear to be essential.

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REFERENCES

- 1 Snell, E. E., Fasella, P. M., Braunstein, A. and Rossi-Fanelli, A. (eds.) (1963) *Chemical and Biological Aspects of Pyridoxal Catalysis*, Pergamon Press, Oxford.
- 2 Anderson, B. M., Anderson, C. D. and Churchich, J. E. (1966) *Biochemistry* **5**, 2893–2900.
- 3 Rippa, M., Spanio, L. and Pontremoli, S. (1967) *Arch. Biochem. Biophys.* **118**, 48–57.
- 4 Ronchi, S., Zapponi, M. C. and Ferri, G. (1969) *Eur. J. Biochem.* **8**, 325–331.
- 5 Shapiro, S., Enser, M., Pugh, E. and Horecker, B. L. (1968) *Arch. Biochem. Biophys.* **128**, 554–562.

- 6 Schnackerz, K. D. and Noltmann, E. A. (1971) *Biochemistry* **10**, 4837–4843
- 7 McKinley McKee, J. S. and Morris, D. L. (1972) *Eur. J. Biochem.* **28**, 1–11
- 8 Gould, K. G. and Engel, P. C. (1980) *Biochem. J.* **191**, 365–371
- 9 Minchiotti, L., Ronchi, S. and Ripa, M. (1981) *Biochim. Biophys. Acta* **657**, 232–242
- 10 Talbot, J.-C., Gross, C., Cosson, M.-P. and Pantaloni, D. (1977) *Biochim. Biophys. Acta* **494**, 19–32
- 11 Raetz, C. R. H. and Auld, D. S. (1972) *Biochemistry* **11**, 2229–2236
- 12 Fischer, E. H., Forrey, A. W., Hedrick, J. L., Hughes, R. C., Kent, A. B. and Krebs, E. G. (1963) in *Chemical and Biological Aspects of Pyridoxal Catalysis* (Snell, E. E., Fasella, P. M., Braunstein, A. and Rossi-Fanelli, A., eds.) pp. 543–562, Pergamon Press, Oxford
- 13 Ritchey, J. M., Gibbons, I. and Schachman, H. K. (1977) *Biochemistry* **16**, 4584–4590
- 14 Whitley, E. J. Jr. and Ginsburg, A. (1978) *J. Biol. Chem.* **253**, 7010–7025
- 15 Hahn, L.-H. E. and Hammes, G. G. (1978) *Biochemistry* **17**, 2423–2429
- 16 Chen, S.-S. and Engel, P. C. (1975) *Biochem. J.* **147**, 351–358
- 17 Chen, S.-S. and Engel, P. C. (1975) *Biochem. J.* **149**, 619–626
- 18 Chen, S.-S. and Engel, P. C. (1974) *Biochem. J.* **143**, 569–574
- 19 Chen, S.-S. and Engel, P. C. (1975) *FEBS Lett.* **58**, 202–206
- 20 Ngo, T. T. and Barbeau, A. (1979) *Int. J. Biochem.* **10**, 937–941
- 21 Ogawa, H. and Fujioka, M. (1980) *J. Biol. Chem.* **251**, 7420–7425
- 22 Frerman, F. E., Andreone, P. and Mielke, D. (1977) *Arch. Biochem. Biophys.* **181**, 508–515
- 23 Dempsey, W. B. and Christensen, H. N. (1962) *J. Biol. Chem.* **237**, 1113–1120
- 24 Piszkiwicz, D. and Smith, E. L. (1971) *Biochemistry* **10**, 4538–4544
- 25 Piszkiwicz, D. and Smith, E. L. (1971) *Biochemistry* **10**, 4544–4552
- 26 Metzler, C. M., Cahill, A. and Metzler, D. E. (1980) *J. Am. Chem. Soc.* **102**, 6075–6082
- 27 Means, G. E. and Feeney, R. E. (1971) *Chemical Modification of Proteins*, Holden-Day, San Francisco
- 28 Metzler, D. E. and Snell, E. E. (1955) *J. Am. Chem. Soc.* **77**, 2431–2437
- 29 Harris, C. M., Johnson, R. J. and Metzler, D. E. (1976) *Biochim. Biophys. Acta* **421**, 181–194
- 30 Cordes, E. H. and Jencks, W. P. (1962) *Biochemistry* **1**, 773–778
- 31 Iwata, C. (1968) *Biochem. Prep.* **12**, 117–121
- 32 Yang, I., Khomutov, R. M. and Metzler, D. E. (1974) *Biochemistry* **13**, 3877–3884
- 33 Bolden, T. C. (1973) M.S. Thesis, Iowa State University
- 34 Engel, P. C. and Dalziel, K. (1969) *Biochem. J.* **115**, 621–631
- 35 Kuroda, T. (1963) *Bitamin* **28**, 21–28
- 36 Khurs, E. H., Metzler, D. E. and Khomutov, R. M. (1978) *Bioorg. Khim.* **4**, 1358–1363
- 37 Dalziel, K. (1962) *Biochem. J.* **84**, 244–254
- 38 Goldin, B. R. and Frieden, C. (1972) *J. Biol. Chem.* **247**, 2139–2144
- 39 Lilley, K. S. and Engel, P. C. (1992) *Eur. J. Biochem.* **207**, 533–540
- 40 Stillman, T. J., Baker, P. J., Britton, K. L. and Rice, D. W. (1993) *J. Mol. Biol.*, in the press
- 41 Gould, K. G. and Engel, P. C. (1982) *Arch. Biochem. Biophys.* **215**, 498–507