A PROTON DONOR–ACCEPTOR FUNCTION OF THE 5′-PHOSPHATE GROUP OF PYRIDOXAL-P IN POTATO PHOSPHORYLASE INFERRED FROM ^{31}P NMR SPECTRA

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1. Introduction

Pyridoxal-5′-phosphate is essential for the activity of prokaryotic and eukaryotic α-glucan phosphorylases (EC 2.4.1.1) [1,2]. For expression of activity, mammalian phosphorylases [2] must be activated either allosterically by 5′-AMP or covalently through phosphorylation of a seryl residue, whereas phosphorylases from potato tubers [3] (and *Escherichia coli* [4,5] or *Klebsiella pneumoniae* [6]) are neither subject to allosteric nor covalent control. Despite these differences there is a high degree of homology in the sequence around the pyridoxal-5′-phosphate binding site [1,5]. This makes potato phosphorylase ideally suited for ^{31}P NMR studies on the role of the cofactor in catalysis.

2. Experimental

2.1. *Materials*

Standard buffer was 50 mM 3-(*N*-morpholino) propane sulfonic acid (Serva, Heidelberg), 50 mM KCl and 2 mM EDTA adjusted to the desired pH with NaOH or HCl. Maltoheptaose was prepared from cyclomaltoheptaose (Sigma) according to [7]. Glycogen, glucose-1-P and other substrates and reagents were obtained from Merck AG or Boehringer.

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2.2. *Enzyme*

Starch phosphorylase was prepared from potato tuber according to [8], but after the second fractionation with $(NH_4)_2SO_4$ the enzyme was dialysed exhaustively against 100 mM arsenate (pH 6.9) to remove primer. The enzyme used was homogeneous according to dodecyl sulfate–polyacrylamide gel electrophoresis. Protein concentrations were determined using $A_{280}^{1\%}$ cm^{-1} = 11.2 [9]. Molar concentrations are based on mol. wt 100 000/monomer [9]. Activity was usually measured with glucose-1-P in the direction of glycogen synthesis [10] or by arsenolysis [11]. The specific activity was ~23 μmol P_i liberated . mg protein^{-1} . min^{-1} at 30°C under assay conditions. The loss of activity in NMR measurements was ⩽ 2%. For ^{31}P NMR measurements, phosphorylase (≈ 400 mg) was collected by precipitation from a stock solution with 3 M $(NH_4)_2SO_4$, centrifuged, redissolved in a small volume of buffer and chromatographed on Sephadex G-25 (2.5 × 30 cm) column.

2.3. *^{31}P NMR*

^{31}P NMR spectra were recorded with a Bruker WH-180 wide-bore superconducting spectrometer in the Fourier transform mode at 72.86 MHz. Enzyme samples (12 ml) corresponding to 1.5–2.5 × 10^{-4} M monomer were placed in 20 mm tubes. A concentric 5 mm NMR tube containing 99% D_2O was used for field/frequency lock. All spectra were recorded with broad-band proton decoupling (0.4 W). Ambient temperature was 27 ± 1°C. In general, a 3000 Hz spectral width was acquired in 4096 data points with 60° pulse angle and 30 μs pulse width. Repetition

time was 0.68 s. The exponential line broadening used prior to Fourier transformation was usually 10 Hz.

3. Results

3.1. *The ionization state of the 5′-phosphate group of pyridoxal-P in potato phosphorylase*

The ionization of the 5′-phosphate, attached to the substituted pyridine ring of the cofactor bound to phosphorylase, is readily measured by ^{31}P NMR spectroscopy [12]. Deprotonation of a model Schiff base of pyridoxal-5′-phosphate with ε-aminocaproate by increasing pH shifts the ^{31}P resonance to lower fields, due to decreased electronic shielding of the ^{31}P nucleus resulting from the expansion of the electron density of the phosphorus by deprotonation [13]. Accordingly we have previously assigned the ^{31}P resonance of non-activated rabbit skeletal muscle glycogen phosphorylase *b* to a mono-protonated species of the 5′-phosphate group of the cofactor at −0.47 ppm relative to the reference triethyl phosphate [12]. Activation of phosphorylase *b* by the allosteric modifier, AMP (AMP-S), in the presence of arsenate or by phosphorylation (phosphorylase *a*) forms a dianionic species at −3.67 ppm with line width $\Delta\nu$ = 70 Hz. Both ionisation states (the mono- and the di-anionic forms) are within a protected site, since they are pH-independent at pH 6.3–8.3 [12]. With potato phosphorylase only one signal for the 5′-phosphate group of the cofactor is observed at −4.5 ppm, with a narrow linewidth of $\Delta\nu$ = 35 Hz, which is pH-independent at pH 6.3–7.8 (fig.1A). Increasing concentrations of arsenate (30–60 mM) shift the ^{31}P resonance upfield and broaden the signal ($\Delta\nu$ = 50 Hz) which becomes sharper again at 60 mM where the linewidth is ~40 Hz and the upfield shift is to −3.75 ppm (fig.1B,C). These signals were assigned in potato phosphorylase in analogy to muscle phosphorylase to dianionic forms of the 5′-phosphate group of pyridoxal-P in a protected hydrophobic environment. The different resonances of the 5′-phosphate group (−4.5 ppm in potato phosphorylase versus −3.67 ppm in activated rabbit muscle phosphorylases) in the absence of arsenate may reflect O–P–O angle distortions, due to different modes of interaction of the dianionic 5′-phosphate group with cationic sites (see [14]) or different degrees of shielding. In the presence of arsenate, the dianionic forms of the 5′-phosphate are in rapid exchange, giving rise to a single ^{31}P resonance with chemical shifts varying varying as a function of arsenate concentration (fig.1B,C).

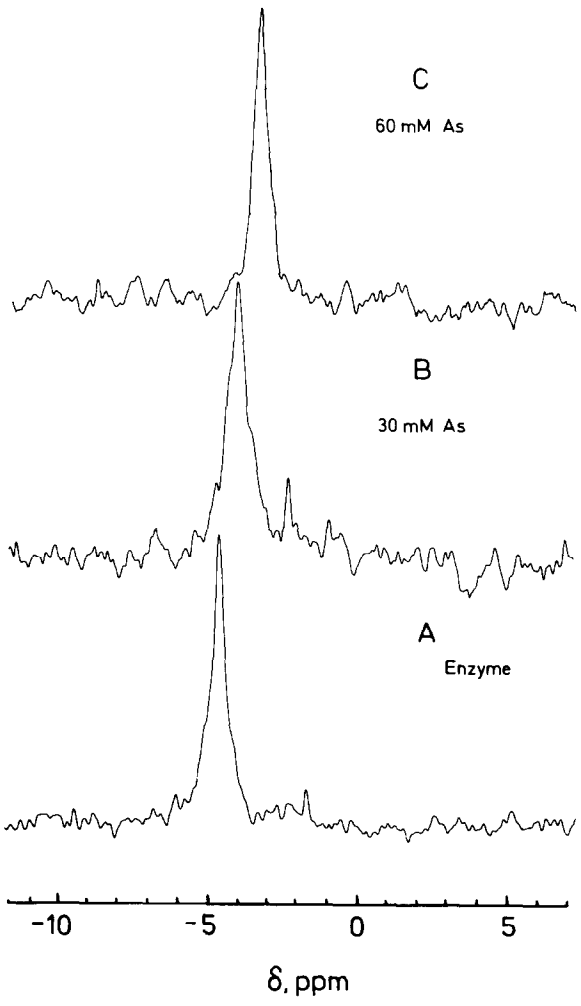

Fig.1. ^{31}P NMR spectra of potato phosphorylase (0.25 mM monomers) in buffer (pH 6.5) with and without arsenate. (A) In the absence of arsenate, 30.712 scans. The chemical shift of pyridoxal-P is −4.5 ppm (relative to triethyl phosphate as external standard) with line width of $\Delta\nu$ = 35 Hz. (B) Same conditions as in (A) but with 30 mM arsenate. The spectrum represents 37.417 scans, δ = −3.98 ppm, $\Delta\nu$ = 50 Hz. (C) Conditions as in (A) and (B) but with 60 mM arsenate. 28.151 scans, δ = −3.75 ppm, $\Delta\nu$ = 40 Hz.

3.2. *Changes in the ionisation state on binding of substrates and substrate analogues*

The X-ray crystallographic evidence indicates that muscle phosphorylase binds glucose (a competitive inhibitor with glucose-1-P), P_i (or arsenate) and glucose-1-phosphate in close proximity (~7 Å) to the phosphate of the cofactor, whereas oligosaccharides (maltoheptaose) and glycogen bind to a site 25–35 Å from the active centre [15–18]. Addition of glucose, which is also a competitive inhibitor with glucose-1-P in potato phosphorylase, (K_i ~85 mM), in amounts up to 150 mM at pH 6.5, resulted in an exchange-broadened ^{31}P resonance signal with a line width of $\Delta\nu \geqslant 80$ Hz and a 1.5 ppm upfield chemical shift (see also [19]). Thus, the 5′-phosphate group of the cofactor becomes partially protonated on binding of glucose (fig.2B). Chemical shift and line broadening suggest that the 5′-phosphate group is in fast exchange on the NMR time scale with an unknown proton donor–acceptor group. It is attractive to speculate that protons are passed via a relay of suitably charged amino acid side chains. This needs to be studied.

The effect of arsenate, which can be substituted for phosphate in catalysis [11] was studied in the ternary complex with glucose (fig.2C), or maltoheptaose (fig.3D) and potato phosphorylase. With 90 mM glucose and 100 mM arsenate, a downfield shift of 0.5 ppm and line narrowing ($\Delta\nu$ = 65 Hz) occurred, indicating that the protons leaving the 5′-phosphate are now in fast exchange with a proton donor–acceptor group which could be arsenate (fig.2C). Chemical shifts and line widths varied with the ratio of glucose:arsenate added, but were independent of pH in the range where the enzyme is catalytically active. Arsenolysis of maltoheptaose yields mainly glucose and arsenate, resulting in a ^{31}P NMR spectrum like that of the expected amount of glucose and arsenate (cf. fig.2C,3D). The free dianionic form is at ~–3.75 ppm and the form in fast exchange at ~–3.44 ppm. Glucose has a low affinity to phosphorylase; high concentrations had therefore to be used, which increase viscosity and could influence protein mobility. As seen in fig.3B, addition of only 1 mM maltoheptaose gives a second resonance at –0.45 ppm with an additional chemical shift of ~4 ppm to higher field. Both ionization states are in slow exchange as indicated by linewidth and separation

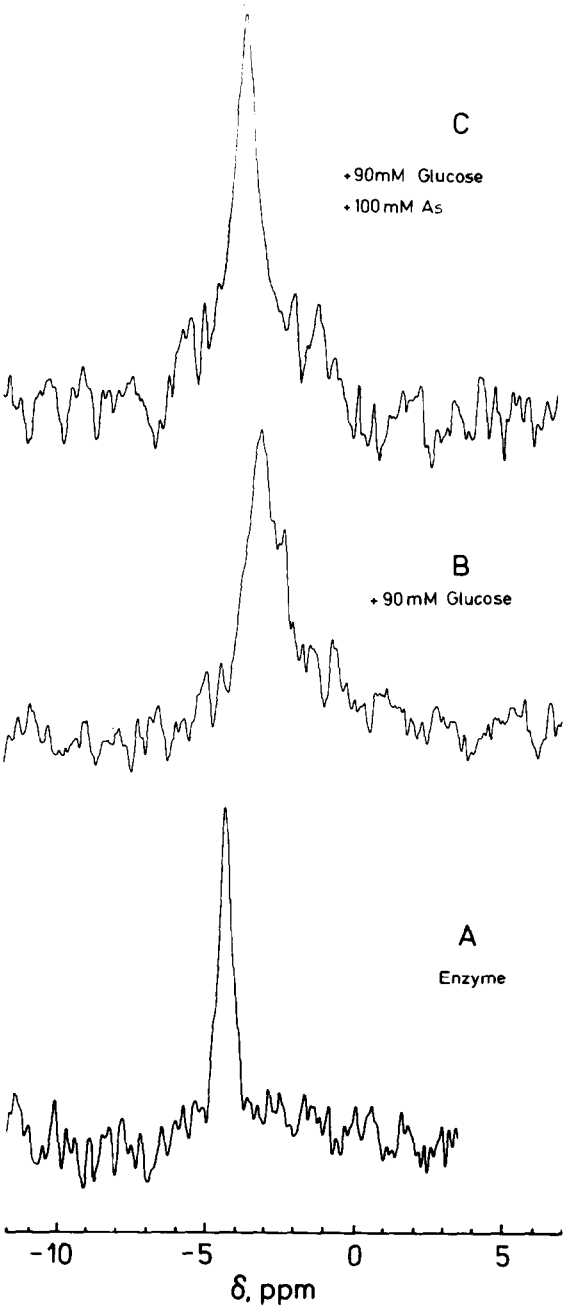

Fig.2. The ionization of pyridoxal-P in the presence of glucose and arsenate. Potato phosphorylase 0.19 mM monomers in buffer (pH 6.5). Conditions for NMR measurements as in fig.1. (A) Enzyme alone, 34.758 scans, pyridoxal-P, δ = –4.5 ppm, $\Delta\nu$ = 35 Hz. (B) With 90 mM glucose, 79.402 scans, δ = –3.0 ppm, $\Delta\nu$ = 80 Hz. (C) With 90 mM glucose and 100 mM arsenate, 73.915 scans, δ = –3.45 ppm, $\Delta\nu$ = 65 Hz.

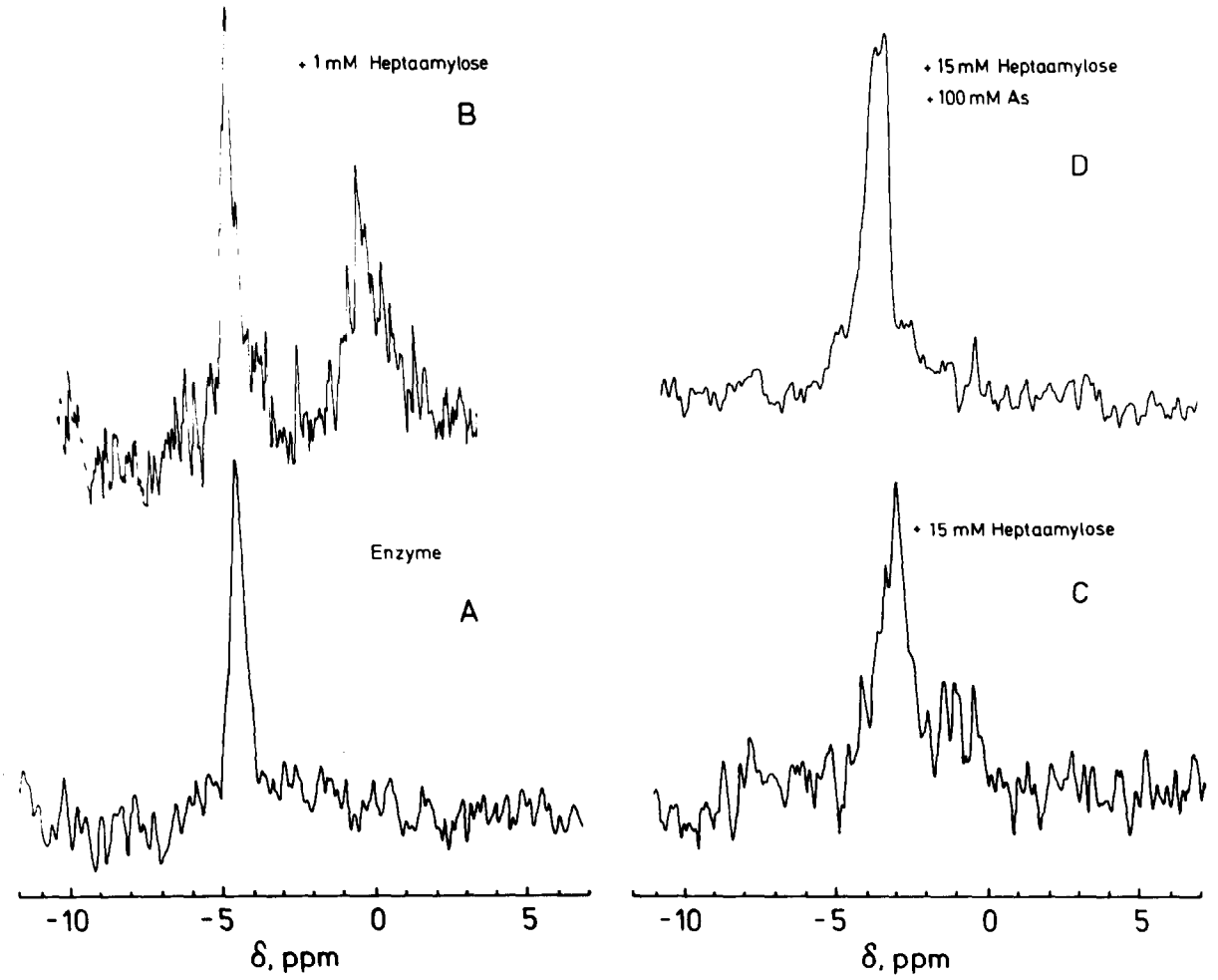

Fig.3. Ionization of pyridoxal-P in the presence of maltoheptaose and arsenate. Potato phosphorylase 0.18 mM monomers in buffer (pH 6.5). Conditions for NMR measurements as in fig.1,2. (A) Enzyme alone, 39.261 scans, $\delta = -4.5$ ppm, $\Delta\nu = 35$ Hz. (B) With 1 mM maltoheptaose, 24.764 scans, pyridoxal-P main peak (left), $\delta = -4.5$ ppm, $\Delta\nu = 35$ Hz, minor signal (right), $\delta = -0.45$ ppm. (C) With 15 mM maltoheptaose, 68.715 scans, $\delta = -3.0$ ppm, $\Delta\nu = 65$ Hz. (D) With 15 mM maltoheptaose and 100 mM arsenate, 58.753 scans, $\delta = \sim -3.75$ ppm (left peak) and ~ -3.44 ppm (right peak).

of signals. With 15 mM maltoheptaose only one signal with $\Delta\nu = 65$ Hz is observed. The higher field resonance had disappeared nearly completely (fig.3C). The chemical shifts for glucose (90 mM) (fig.2B) and maltoheptaose (15 mM) (fig.3C) are similar, with the exception, however, of a significant line narrowing in the case of the latter. In both cases, with glucose and maltoheptaose, the chemical shifts are pH-independent at pH 6.2–6.9, where potato phosphorylase has 100–75% activity. At pH 7.6 with ~40% of the activity remaining, the partially protonated species decreases.

4. Conclusion

The active form of the cofactor in muscle [12], *E. coli* [20] and potato phosphorylase is the dianionic 5′-phosphate group (fig.1A). The dianionic form is pH-independent, suggesting that it is still shielded in a hydrophobic environment.

On binding maltoheptaose or glucose to potato phosphorylase, the 5′-phosphate group becomes partially protonated as indicated by the chemical shift to higher field (to −3.0 ppm) (see fig.2B,3C). Protonation is pH-independent in the range of maximal enzymatic activity. Cyclodextrins (cyclomaltohexaose or cyclomaltoheptaose), sucrose, 2-deoxy-D-glucose or D-glucal did not promote protonation. The behaviour of a 'classical' pyridoxal-P-dependent enzyme, *E. coli* D-serine dehydratase, is different: In that case, the phosphate group of the cofactor behaved like a model Schiff base with pK 6.4 indicating location in a polar environment, and addition of substrates or substrate analogues made the signal pH-independent, presumably as a consequence of salt bridge formation [21]. At low concentrations of oligosaccharide ($\lesssim$ 1 mM), two separated ^{31}P resonances in slow exchange on the NMR time scale were observed (fig.3B). Since the linewidth of the resonance at −0.45 ppm is considerably broadened, but $\Delta\nu$ of the original peak at −4.5 ppm remains unchanged even at comparable concentrations of the two forms, an additional fast exchange broadening for the former resonance at −0.45 ppm has to be considered (fig.3B). With increasing concentrations of maltoheptaose the form represented by the exchange broadened signal increases with a concomitant lower field shift, suggesting fast exchange among different protonated forms (fig.3C) (see also [19]). If potato phosphorylase should have a storage site like muscle phosphorylase, the concentration dependence might be interpreted in the light of kinetic studies on oligosaccharide binding to rabbit muscle phosphorylase *a* [17] which suggested that a storage site with high affinity has to be saturated first, before oligosaccharides (and glycogen) can bind at the active site.

With glucose or maltoheptaose and arsenate and potato phosphorylase we could demonstrate a fast equilibrium between protonated and deprotonated (dianionized) forms (fig.2C,3D). Similar ^{31}P NMR data have recently been obtained with *E. coli* maltodextrin phosphorylase and glucose and arsenate (H.W.K., (1979) unpublished experiments). The rather similar changes of the ionization of the 5′-phosphate group of pyridoxal-P at nearly saturating concentrations of glucose and maltoheptaose and arsenate are puzzling (see fig.2C,3D), because glucose and maltoheptaose bind to different sites. The former binds to the glucose-1-P site which is near the 5′-phosphate of pyridoxal-P and the latter to a polysaccharide (storage?) site (see [17,18]). Obviously, the protonation equilibrium in the presence of arsenate and glucose cannot reflect attack by arsenate on a 1,4-α-glycosidic bond. But the similar protonation–deprotonation equilibria with glucose and maltoheptaose and arsenate are readily reconciled if one considers a common glucosyl transfer site. Thus, assuming that this equilibrium reflects proton transfer in catalysis and not merely structural rearrangements, it would represent the first evidence supporting a function of the 5′-phosphate group of the cofactor as a proton donor–acceptor in catalysis, which was postulated by one of us more than 10 years ago [22]. In support of that is the argument that cyclodextrins which do not have a free hydroxyl group are ineffective, although they are bound to potato phosphorylase [23]. Moreover, the experimental evidence presently available suggests that proton flux only occurs with a substrate anion. Sulfate and methylphosphonate were ineffective. Experiments with [^{13}C]glucose-1-P are underway to study the change in the substrate on binding and at protonation–deprotonation equilibrium and to estimate proton-transfer rates.

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References

[1] Titani, K., Koide, A., Ericsson, L. H., Kumar, S., Hermann, J., Wade, R. D., Walsh, K. A., Neurath, H. and Fischer, E. H. (1978) Biochemistry 17, 5680–5695.

[2] Graves, D. J. and Wang, J. H. (1972) in: The Enzymes (Boyer, P. D. ed) 3rd edn, vol. 7, pp. 435–482, Academic Press, London, New York.

[3] Nakano, K., Wakabayashi, S., Hase, T., Matsubara, H. and Fukui, T. (1978) J. Biochem. (Tokyo) 83, 1085–1094.
[4] Schwartz, M. and Hoffnung, M. (1967) Eur. J. Biochem. 2, 132–145.
[5] Schächtele, K.-H., Schiltz, E. and Palm, D. (1978) Eur. J. Biochem. 92, 427–435.
[6] Linder, D., Kurz, G., Bender, H. and Wallenfels, K. (1976) Eur. J. Biochem. 70, 291–303.
[7] Hu, H.-Y. and Gold, A. M. (1975) Biochemistry 14, 2224–2230.
[8] Staerk, J. and Schlenk, H. (1967) Biochim. Biophys. Acta 146, 120–128.
[9] Franken, K.-D., Keilich, G. and Husemann, E. (1972) Die Stärke 24, 37–42.
[10] Feldmann, K., Zeisel, H. and Helmreich, E. J. M. (1976) Eur. J. Biochem. 65, 285–291.
[11] Helmreich, E. J. M. and Cori, C. F. (1964) Proc. Natl. Acad. Sci. USA 51, 131–138.
[12] Feldmann, K. and Hull, W. E. (1977) Proc. Natl. Acad. Sci. USA 74, 856–860.
[13] Martinez-Carrion, M. (1975) Eur. J. Biochem. 54, 39–43.
[14] Feeny, J., Birdsall, B., Roberts, G. C. K. and Burgen, A. S. V. (1977) in: NMR in Biology (Dwek, R. A. et al. eds) pp. 111–124, Academic Press, London, New York.
[15] Weber, I. T., Johnson, L. N., Wilson, K. S., Yeates, D. G. R., Wild, D. L. and Jenkins, J. A. (1978) Nature 274, 433–437.
[16] Sygusch, J., Madsen, N. B., Kasvinsky, P. J. and Fletterick, R. J. (1977) Proc. Natl. Acad. Sci. USA 74, 4757–4761.
[17] Kasvinsky, P. J., Madsen, N. B., Fletterick, R. J. and Sygusch, J. (1978) J. Biol. Chem. 253, 1290–1296.
[18] Kasvinsky, P. J., Madsen, N. B., Sygusch, J. and Fletterick, R. J. (1978) J. Biol. Chem. 253, 3343–3351.
[19] Hoerl, M., Feldmann, K., Schnackerz, K. D. and Helmreich, E. J. M. (1979) Biochemistry 18, 2457–2464.
[20] Palm, D., Schächtele, K.-H., Feldmann, K. and Helmreich, E. J. M. (1979) FEBS Lett. 101, 403–406.
[21] Schnackerz, K. D., Feldmann, K. and Hull, W. E. (1979) Biochemistry 18, 1536–1539.
[22] Kastenschmidt, L. L., Kastenschmidt, J. and Helmreich, E. J. M. (1968) Biochemistry 7, 3590–3608.
[23] Kokesh, F. C. and Kakuda, Y. (1977) Biochemistry 16, 2467–2473.