Thermal denaturation pathway of starch phosphorylase from *Corynebacterium callunae*: Oxyanion binding provides the glue that efficiently stabilizes the dimer structure of the protein

RICHARD GRIEBLER,¹ SABATO D'AURIA,² FABIO TANFANI,³ AND BERND NIDETZKY¹

¹Division of Biochemical Engineering, Institute of Food Technology, Universität für Bodenkultur Wien (BOKU), Muthgasse 18, A-1190 Wien, Austria

²Institute of Protein Biochemistry and Enzymology, C.N.R., Via Marconi, 10, 80125 Naples, Italy

³Institute of Biochemistry, Medical School, University of Ancona, Via Ranieri, 60131 Ancona, Italy

(RECEIVED October 19, 1999; FINAL REVISION March 16, 2000; ACCEPTED March 16, 2000)

Abstract

Starch phosphorylase from *Corynebacterium callunae* is a dimeric protein in which each mol of 90 kDa subunit contains 1 mol pyridoxal 5'-phosphate as an active-site cofactor. To determine the mechanism by which phosphate or sulfate ions bring about a greater than 500-fold stabilization against irreversible inactivation at elevated temperatures ($\geq 50^\circ\text{C}$), enzyme/oxyanion interactions and their role during thermal denaturation of phosphorylase have been studied. By binding to a protein site distinguishable from the catalytic site with dissociation constants of $K_{\text{sulfate}} = 4.5\text{ mM}$ and $K_{\text{phosphate}} \approx 16\text{ mM}$, dianionic oxyanions induce formation of a more compact structure of phosphorylase, manifested by (a) an increase by about 5% in the relative composition of the α -helical secondary structure, (b) reduced $^1\text{H}/^2\text{H}$ exchange, and (c) protection of a cofactor fluorescence against quenching by iodide. Irreversible loss of enzyme activity is triggered by the release into solution of pyridoxal 5'-phosphate, and results from subsequent intermolecular aggregation driven by hydrophobic interactions between phosphorylase subunits that display a temperature-dependent degree of melting of secondary structure. By specifically increasing the stability of the dimer structure of phosphorylase (probably due to tightened intersubunit contacts), phosphate, and sulfate, this indirectly (1) preserves a functional active site up to $\approx 50^\circ\text{C}$, and (2) stabilizes the covalent protein cofactor linkage up to $\approx 70^\circ\text{C}$. The effect on thermostability shows a sigmoidal and saturable dependence on the concentration of phosphate, with an apparent binding constant at 50°C of $\approx 25\text{ mM}$. The extra stability conferred by oxyanion–ligand binding to starch phosphorylase is expressed as a dramatic shift of the entire denaturation pathway to a $\approx 20^\circ\text{C}$ higher value on the temperature scale.

Keywords: α -glucan phosphorylase; denaturation mechanism; oxyanion ligand; phosphate stabilization

Unlike a great number of α -D-glycoside hydrolases, α -1,4-D-glucan phosphorylases catalyze the degradation of α -1,4-D-glucan molecules by using phosphate rather than water as an acceptor of the transferred glucosyl moiety, as shown in Equation 1 where N is the degree of polymerization of the α -1,4-D-glucan.

Therefore, interactions between the enzyme and the substrate phosphate play an essential role in the catalytic cycle of phosphorylase and contribute to binding energy in the ground state and the transition state of the reaction (Johnson et al., 1990; Schinzel & Drueckes, 1991; Johnson, 1992; Watson et al., 1997, 1999). However, apart from typical enzyme/substrate interactions, a number of other important interactions between phosphorylase and phosphate are known and have been studied on a molecular level: (1) phosphate functions as general acid that protonates the glycosidic oxygen of the substrate and thereby assists leaving-group

Reprint requests to: Bernd Nidetzky, Institut für Lebensmitteltechnologie, BOKU, Muthgasse 18, A-1190 Wien (Vienna), Austria; e-mail: nide@edv2.boku.ac.at.

Abbreviations: amide I', amide I band in $^2\text{H}_2\text{O}$ medium; CD, circular dichroism; FTIR spectroscopy, Fourier-transform infrared spectroscopy; Glc 1-P, α -D-glucose 1-phosphate; P_i , inorganic phosphate; PLP, pyridoxal 5'-phosphate; StP, starch phosphorylase from *Corynebacterium callunae*; T_m , melting temperature.

departure (Johnson et al., 1990; Palm et al., 1990; Watson et al., 1999); (2) by serving as a Brønsted or electrophilic catalyst, the phosphate moiety of the protein-bound cofactor pyridoxal 5'-phosphate is essential for enzyme activity (Madsen & Withers, 1986; Johnson et al., 1990; Palm et al., 1990; Stirtan & Withers, 1996; Bartl et al., 1999; Watson et al., 1999); (3) enzyme/phosphate interactions contribute to the driving force for binding of the allosteric effectors, AMP and glucose 6-phosphate, to regulated phosphorylases (Sprang et al., 1988, 1991; Barford & Johnson, 1989; Barford et al., 1991; Johnson, 1992; Johnson et al., 1993); (4) covalent phosphorylation in the N-terminal domain regulates activity of phosphorylases from mammals (Sprang et al., 1988; Goldsmith et al., 1989; Barford et al., 1991) and yeast (Lin et al., 1996; Rath et al., 1996). Apart from these many roles of protein/phosphate interactions pertaining to phosphorylase function, interactions between phosphorylase and the phosphate ion could contribute to the stabilization of functional and regulatory sites of the enzyme, the phosphorylase dimer or the structural core of the protein subunit. That has not been studied in detail so far.

The present paper deals with phosphorylase/phosphate interactions related to thermostability. For our studies, we chose starch phosphorylase (StP) from the mesophilic bacterium *Corynebacterium callunae*, which, like the structurally well-characterized maltodextrin phosphorylase from *Escherichia coli* (Watson et al., 1997, 1999; O'Reilly et al., 1998, 1999), belongs to the nonregulated class of α -1,4-D-glucan phosphorylases, whose enzyme activities are subject to control neither by covalent phosphorylation nor allosteric effectors. We describe experiments that are aimed at determining the mechanism by which oxyanions such as phosphate or sulfate (1) bring about a greater than 500-fold stabilization of StP against irreversible inactivation in the temperature range 50 to 70 °C and (2) increase the melting point of the structural core of the protein by almost 20 °C. Although ligand-induced enzyme stabilization has been documented in a number of instances, a clear causal relation has rarely been worked out between the amount of binding energy derived from the protein/ligand interactions, the resulting conformational changes in the protein, and their effects in the denaturation pathway.

The results show that by binding to a saturable, moderate-affinity oxyanion site that is clearly distinguishable from the phosphate binding site in the active site of StP, phosphate, and sulfate induce a number of structural modifications in StP, best summarized as "conformational tightening," which translate into a dramatic stabilization of the dimer structure of the enzyme. Thereby, dissociation of the cofactor PLP is inhibited, and irreversible losses of enzyme activity are prevented in a very efficient manner even at temperatures as high as 60–70 °C. The structural integrity of the phosphorylase subunit at 30 °C or greater appears to be tightly coupled to the stability of the dimer, and depending on temperature the dimer occurs in enzymatically active and inactive forms. Native and nonnative dimers are stabilized by phosphate and sulfate via specific binding interactions, and at very high temperatures an additional lyotropic effect of these oxyanions seems to be important. The comparison of the stabilities of StP (this work), maltodextrin phosphorylase (Griebler et al., 2000), and other α -glucan phosphorylases (Griebler et al., 1998) makes it clear that despite the similar overall fold of individual α -glucan phosphorylases (Newgard et al., 1989; Johnson, 1992; Watson et al., 1997), the stabilities of the native dimers, the active sites, and the structural cores of the protomers can vary by as much as ≈ 15 °C among family members isolated from mesophilic sources. To the best of our

knowledge, the magnitude of the phosphate effect on the thermostability of StP is not precedent with other proteins for which stabilization by phosphate has been reported.

Results and discussion

Binding of phosphoryl ligands to the active site of StP

A common observation with α -glucan phosphorylases is that on addition of phosphate ions to free enzyme, PLP fluorescence is quenched, reflecting binding of phosphate to the active site of phosphorylase (Cortijo et al., 1971; Honikel & Madsen, 1973). In contrast, the cofactor fluorescence of StP was enhanced when P_i was added (Fig. 1A). The effect was weak but significant, giving a maximum fluorescence increase of about 40% compared with the

Fig. 1. Phosphoryl-ligand binding to StP determined by titration of the PLP fluorescence. Experiments were done at 30 °C in 50 mM triethanolamine buffer, pH 6.9, using 450 μ g/mL phosphorylase. **A:** Fluorescence enhancement by binding of phosphate. **B:** Fluorescence quenching by binding of Glc 1-P. I and I_0 are the intensities of the PLP fluorescence in the presence and absence of ligand. The lines are the fits of the data to the hyperbolic equation of a single-ligand binding isotherm.

control that did not contain P_i . Conversely, Glc 1-P efficiently quenched the PLP fluorescence, by almost fivefold in the presence of excess (20–30 mM) of ligand (Fig. 1B). Albeit in opposite directions, the effects of P_i and Glc 1-P on PLP fluorescence showed saturation with increasing ligand concentration. Therefore, it was possible to calculate dissociation constants (K_d) for binary enzyme complexes with P_i and Glc 1-P of 7.6 ± 0.7 mM and 0.49 ± 0.03 mM, respectively. The K_d value for the binding of Glc 1-P is in good agreement with the corresponding Michaelis constant (K_m) of 0.7 mM (Weinhäusel et al., 1997). Note that K_m in a rapid-equilibrium random bi bi kinetic mechanism, such as that of α -glucan phosphorylase (Engers et al., 1970; Gold et al., 1970), reflects K_d for this substrate. The K_d value for phosphate is ~ 6.5 -fold greater than the corresponding K_m value, which is 1.2 mM (Weinhäusel et al., 1997). However, substrate binding by phosphorylase in phosphorylase is known to be synergistic (Engers et al., 1970; Becker et al., 1994), and kinetic experiments with StP revealed an interaction coefficient, α , of ~ 0.13 (R. Griebler & B. Nidetzky, unpubl. obs.). Therefore, in the absence of the α -glucan substrate, phosphate binding should equal the expression K_m/α , which is $1.2/0.13 = 7$, and agrees with the results of fluorescence titration. Sulfate, a weak competitive inhibitor with respect to phosphate ($K_i = K_d > 50$ mM), did not affect the fluorescence of PLP to any measurable extent when added to StP in concentrations of 10–100 mM. The increase of PLP fluorescence in the presence of phosphate could reflect small conformational rearrangements in the active site on phosphate binding and indicate slight differences in the binding of the phosphoryl group in Glc 1-P and phosphate, as found by comparing structures of glycogen phosphorylase ligated with phosphate and a cofactor-substrate analog (Sprang et al., 1992).

Binding of phosphate and sulfate to a second oxyanion site in StP

This was determined by making use of the chemical quenching by iodide of the cofactor fluorescence in StP. Figure 2A shows iodide quenching of PLP fluorescence in the presence of Glc 1-P. Up to an iodide concentration of ~ 0.25 M, PLP fluorescence increased when increasing amounts of iodide were added to the protein solution. These data reflect the competition between iodide and Glc 1-P for binding to the active site of the enzyme and indicate that iodide quenches the PLP fluorescence less efficiently than does Glc 1-P. When the iodide concentration was increased further, PLP fluorescence decreased, reflecting its chemical quenching by iodide ions. In marked contrast to these observations, phosphate or sulfate strongly inhibited the quenching of PLP fluorescence by iodide, relative to a control that did not contain oxyanion. This is shown as a Stern–Volmer plot in Figure 2B. Independent of the presence of oxyanion, the individual curves are concave upward, probably indicating that quenching by iodide results from collisional quenching and additional static quenching brought about by binding effects (see also Fig. 2A). However, the effects of phosphate and sulfate are nevertheless clear (Fig. 2B). In particular, the effect of sulfate is interesting because sulfate binds poorly to the catalytic site of StP ($K_i > 50$ mM). Therefore, this implies that the protection of the cofactor fluorescence against chemical quenching by iodide observed in the presence of 4 mM sulfate must originate from interactions between StP and sulfate, which are different from the interactions of this oxyanion with the catalytic site of StP. Two additional pieces of evidence provide strong sup-

port for the suggestion that the inhibition of iodide quenching of the cofactor fluorescence results from binding of oxyanions to a distinct “site” that is not coincident with the phosphate binding site in the active site of StP. (1) The effect of phosphate or sulfate showed a saturable dependence on the oxyanion concentration, and no competition was observed between Glc 1-P and sulfate regarding the effect of iodide on cofactor fluorescence; (2) as shown below, the dissociation constants of sulfate and phosphate for binding to the catalytic site are significantly different from those for binding to the oxyanion site in StP.

The fact that the iodide concentration, at which the quenching of the cofactor fluorescence of StP is 50%, is shifted by up to 160 and 120 mM upon addition of phosphate and sulfate, respectively, has been used to determine the dissociation constants (K_d) of the binary complexes between StP and these oxyanions, bound to the proposed oxyanion site of the protein. Results are shown as a double-reciprocal plot in Figure 2C. The K_d values at pH 6.9 and 30 °C were obtained from nonlinear fits of the original data and are 16 ± 4 mM for phosphate and 4.5 ± 0.5 mM for sulfate. (Scatchard analysis gives identical values for K_d . However, the number of ligand binding sites could not be determined with sufficient accuracy because of the moderate affinity of oxyanions for binding to StP.) They are useful for the classification of the oxyanion site as a moderate-affinity binding site to which phosphate binds approximately twofold less strongly than it binds to the active site of StP unliganded with α -glucan. By contrast, sulfate binds with an at least 10-fold greater affinity to the oxyanion site than it binds to the active site of StP.

Conformational “tightening” induced by the binding of oxyanions

The original absorbance spectra of StP in the presence of 50 or 100 mM P_i are shown in Figure 3A. The spectra reveal the same amide I' band position ($1,651\text{ cm}^{-1}$) and bandwidth, and almost the same residual amide II band ($1,549.6\text{ cm}^{-1}$) intensity. In the presence of 2 mM P_i , the amide I' band is slightly broader and located at $1,649.6\text{ cm}^{-1}$, and the residual amide II band ($1,552\text{ cm}^{-1}$) intensity is lower than in the other two samples (Fig. 3A). These findings indicate that P_i affects in some way the structure of the enzyme. In particular, when a protein is dissolved in a $^2\text{H}_2\text{O}$ medium, its amide II band ($1,600\text{--}1,500\text{ cm}^{-1}$) intensity decreases depending on the extent of $^1\text{H}/^2\text{H}$ exchange (Osborne & Navedryk-Viala, 1982). The fact that all samples show a residual amide II band indicates that the solvent cannot reach all protein segments. Moreover, the higher amide II band intensity found in the presence of 50 or 100 mM P_i than in 2 mM P_i indicates that a high concentration of P_i decreases the accessibility of the solvent to StP, probably by inducing a more compact protein structure.

Information on StP secondary structure has been obtained from the deconvolved spectra in which the amide I' components ($1,700\text{--}1,600\text{ cm}^{-1}$) are characteristic of a particular secondary structure (Byler & Susi, 1986; Jackson & Mantsch, 1991, 1992; Arrondo et al., 1992; Fernandez-Ballester et al., 1992; Muga et al., 1993; Banuelos et al., 1995). The deconvolved spectra of the enzyme in the presence of 2, 50, and 100 mM P_i are compared in Figure 3B. They reveal marked differences for spectra recorded in 2 mM P_i , and in 50 or 100 mM P_i . In particular, high P_i concentration induces an upshift in wavenumber of the α -helix band from $1,654.7$ to $1,655.6$ and of the β -sheet band from $1,639.4$ to $1,640.6\text{ cm}^{-1}$.

Fig. 2. Iodide quenching of the cofactor fluorescence in StP for the determination of protein/ligand interactions at the oxyanion site. **A:** Effect of iodide on the PLP fluorescence of the binary complex between StP and Glc 1-P. **B:** Effect of sulfate and phosphate on iodide quenching. F_0 and F are the fluorescence intensities of PLP in the absence and presence of iodide. **C:** Double-reciprocal plots for the determination of dissociation constants of the binary complex between StP and phosphate (full squares) and sulfate (full circles). The concentration of iodide (C_I) at which $F_0/F = 2$ was determined by extrapolation using a fifth-order polynomial fit to the original data, as shown in **B**. The value of (Δiodide) is the difference of C_I values in the presence and absence of oxyanion. Experiments were done at 30 °C in 50 mM triethanolamine buffer, pH 6.9, at a constant ionic strength of 1.0 with 450 $\mu\text{g/mL}$ phosphorylase.

This finding is in accordance with the lower accessibility of the solvent to the protein at high P_i concentration, because lower $^1\text{H}/^2\text{H}$ exchange is usually manifested by higher α - and β -sheet band positions in the FTIR spectrum. Besides bandshift, the high P_i concentrations induce significant increases of the α -helix band and, concomitantly, a decrease of the β -sheet band. Therefore, this indicates a modification of the protein secondary structure composition induced by P_i . Estimates of the α -helical secondary structure content of StP have been made from measurements by CD (spectra not shown). StP contains about 48% α -helix in the absence of phosphate. When 50 mM P_i are added, this figure increases to $\approx 57\%$. The FTIR data corroborate this “ α -helix-forming” effect of phosphate, indicating an increase by $\sim 5\%$ in the α -helical structure of the enzyme in 50 or 100 mM P_i , compared with StP in 2 mM P_i .

The other amide I' components show almost the same band intensity and position in all samples. The bands at 1,679.4 and 1,669 cm^{-1} are probably due to turns (Banelos et al., 1995), while the 1,688.3 cm^{-1} band, beside the 1,640.6 and 1,639 cm^{-1} bands, can also be assigned to β -structure (Banelos et al., 1995). The 1,624.1 cm^{-1} band could be due to intermolecular interactions, to an unusually strongly hydrogen-bonded β -sheet or to a particularly solvent-exposed β -sheet (Jackson & Mantsch, 1992). The 1,549.4 cm^{-1} peak represents the residual amide II band, while the other bands displayed in Figure 3B may be assigned to amino acid side-chain absorption (Chirgadze et al., 1975).

The thermostability of StP is increased dramatically upon oxyanion ligand binding

When dissolved in Tris, Mes, or triethanolamine buffers (50 mM, pH 6.9) but without added phosphate (≤ 0.1 mM), StP was inactivated within a few hours at 30 °C. Inactivation with time was irreversible. However, it appeared as a first-order reaction. On addition of P_i , stability of StP at 30 °C and at elevated temperatures up to 70 °C was increased dramatically. Stabilization brought about by 50 mM P_i at 50 °C (50 mM Tris, pH 6.9) was at least 500-fold compared with a control that did not contain phosphate. The extra stability conferred by P_i was dependent on the phosphate concentration and the incubation temperature. In the range 30 to 55 °C, phosphate concentrations of 1 to 40 mM stabilized the enzyme markedly. At 50 °C, the half-life time of StP showed a dependence on the phosphate concentration that appears sigmoidal and saturable (Fig. 4). The half-saturation constant for the binding of phosphate was estimated from Figure 4 and is ~ 25 mM.

When the temperature was raised to 60 or 70 °C, high concentrations of P_i (> 0.3 M) were needed to observe stabilization of StP. The dependence on the P_i concentration of the half-life time of enzyme activity was concave upward (Fig. 4, inset). This finding probably indicates that at very high temperatures, phosphate stabilizes StP by an additional lyotropic (salting-out) anion effect (Baldwin, 1996). However, this effect was quite specific for phosphate and sulfate. Other strong lyotropic anions such as citrate, for example, did not stabilize StP at 60 °C or greater, even in concentrations of 0.5–1.5 M and in the presence of 50 mM phosphate.

With regard to stabilization of StP, sulfate could fully replace phosphate at all temperatures between 30 and 70 °C. Structural analogs of phosphate showed significant stabilization of StP up to a maximum temperature of 55 °C in the following order of effectiveness: arsenate pyrophosphate $\approx$ phosphite $>$ thiophosphate. However, at 55 °C, phosphate and sulfate stabilized about 50-fold

Fig. 3. Conformational changes in StP induced by oxanion binding. (A) Original and (B) deconvoluted spectra of StP in $^2\text{H}_2\text{O}$, and at 20°C and p^2H 6.8. Continuous, dashed, and dotted lines refer to StP in 2, 50, and 100 mM P_i , respectively.

better than arsenate. A number of other anionic compounds did not affect the stability of StP at 50°C : phosphorylated sugars (≤ 50 mM) such as the substrate Glc 1-P, glucose 6-phosphate, or fructose 6-phosphate; nucleotides (≤ 20 mM) such as AMP, ADP, ATP; other oxanions such as borate and carbonate. The common structural features of oxanions that efficiently stabilize StP are, therefore, four oxygen atoms, tetrahedral coordination of oxygen atoms around the central atom, formal charge of -2 (sulfate) at pH 6.9, and ion radii between 4.5 Å (sulfate) and 4.9 Å (arsenate). Glc 1-P competes with phosphate for binding to the catalytic site of StP, but it did not inhibit the stabilizing effects of phosphate or sulfate. (The stabilizing effect of phosphate decreased dramatically with decreasing pH between pH 7 and 6. This *could* be an indication that HPO_4^{2-} is the form of phosphate that brings about stabilization of StP.) Therefore, this finding provides strong support for the

suggestion that the interactions with phosphoryl ligands at the catalytic site of StP are not responsible for conferring extra stability to the protein. Cationic buffers, such as Tris or imidazol, and monovalent anions, such as the weakly lyotropic chloride or acetate, brought about a marked destabilization of the phosphorylase and counteracted the effect of P_i .

Temperature dependence of kinetic parameters and reversible loss of a functional active site

The apparent kinetic parameters in the direction of α -glucan phosphorylase were determined with phosphate as the varied substrate in the range 30 to 70°C . The values of the turnover number (k_{cat}) showed a concave-upward (exponential) dependence on the temperature between 30 and 45°C (Table 1). The K_m ($= K_d$) for

Fig. 4. Ligation with oxyanions stabilizes StP against irreversible inactivation. Experiments were done in 50 mM Tris buffer, pH 6.9, at 50 and 70 °C (inset), using a protein concentration of 50 $\mu\text{g}/\text{mL}$.

phosphate increased from 14 to 17 mM between 30 and 45 °C, indicating a very small temperature dependence of the dissociation constant of phosphate in this temperature range. At 55 °C, however, there was a marked increase in K_m for phosphate (≈ 40 mM). The optimum value of k_{cat} was observed between 45 and 55 °C ($\approx 120 \text{ s}^{-1}$), and the turnover number decreased sharply at higher temperatures. Because the enzyme was completely stable, i.e., not inactivated irreversibly during the time period (15 min) of the assay up to 70 °C, the decrease in k_{cat} at 60 °C ($\approx 104 \text{ s}^{-1}$), 65 °C ($\approx 64 \text{ s}^{-1}$), and 70 °C ($\approx 6 \text{ s}^{-1}$), compared with k_{cat} at 45–55 °C, reflects reversible inactivation of StP. A number of experimental observations suggest that structural modifications of StP leading to a reversible loss of enzyme activity are small and do not affect the oligomeric state of the enzyme. (1) By using difference FTIR (cf. Fig. 10) small changes in the structure of StP were detected in the range 45 to 55 °C. (2) No release of PLP took place at ≈ 55 °C in the presence of 50 mM P_i . (3) Renaturation of reversibly inac-

tivated StP occurred within the dead time of the activity assay. However, the association of phosphorylase holo-protomers is a slow process at 30 °C. Even in the presence of excess of PLP so as to make cofactor binding to the apo-protomers not rate limiting, it requires at least several minutes to be complete (Shaltiel et al., 1969; Shimomura et al., 1980). Therefore, reversible inactivation is not due to dissociation of subunits.

We would like to distinguish between the contributions to extra stability against reversible and irreversible thermal denaturation made by the binding of ligands to the oxyanion site in StP. To this end, kinetic measurements were carried out in the direction of α -glucan synthesis, using sulfate as the stabilizing ligand. Results are shown in Figure 5. In the absence of sulfate, the temperature profile of k_{cat} showed an optimum value at ~ 35 °C, reflecting irreversible denaturation of StP above 35 °C during the time of the assay (10 min). In contrast, the maximum value of the turnover number in the presence of 20 mM sulfate was observed at 45 °C. [The small difference in the optimum temperatures for phosphorylase (≈ 50 °C) and synthesis (45 °C) is probably due to the fact that saturation with α -glucan at high temperatures is difficult to achieve in synthesis direction. The K_m values for the α -glucan at 30 °C are 10-fold greater for the synthesis direction, compared with the phosphorolysis direction (Weinhäusel et al., 1997).] It reflects denaturation of the enzyme above 45 °C that is fully reversible upon cooling to room temperature. Therefore, binding of phosphate or sulfate at the oxyanion site chiefly stabilizes against the irreversible inactivation. If any, its effect on reversible denaturation is small.

Table 1. Temperature dependence of kinetic parameters of StP in phosphorolysis at pH 6.9^a

<i>T</i> (°C)	<i>k</i> _{cat} (s ⁻¹)	<i>K</i> _m (mM) ^{b,c}	<i>k</i> _{cat} / <i>K</i> _m (mM ⁻¹ s ⁻¹)
30	45 ± 2	14 ± 1	3.2
40	86 ± 3	16 ± 2	5.4
45	120 ± 4	17 ± 2	7.0
50	127 ± 5	37 ± 6	3.4

^aDetermined in phosphate-buffered solution.

^b K_m for phosphate.

^c K_m in phosphate buffer is ~ 10 times the K_m in Tris or triethanolamine buffer (Weinhäusel et al., 1997).

Conformational changes, cofactor and subunit dissociation, and aggregation at elevated temperature

Changes in the absorbance spectrum of StP were monitored during thermal denaturation. At 30 °C, the protein showed a characteristic

Fig. 5. Temperature dependence of the turnover number of StP in direction of α -glucan synthesis. Experiments were done in 50 mM triethanolamine buffer, pH 6.9, in the absence (open squares) and presence (full circles) of 20 mM sulfate, using a protein concentration of 55 μ g/mL. Lines are drawn to indicate the trend of the data.

absorbance peak at 330–332 nm, reflecting the enzyme-bound PLP. In the absence of phosphate, this peak disappeared on increasing the temperature to 45 °C, and an increase in the absorbance at 405 nm was observed. This indicates that PLP was released into solution. Between 47 and 52 °C, the absorbance at 281 nm increased with concomitant appearance of turbidity, reflecting aggregation and precipitation of StP. Aggregation of StP at $\approx$ 52 °C was confirmed by light-scattering measurements. A good correlation was found (Fig. 6) between irreversible loss of enzyme activity and the increase in the absorbance at 281 nm, relative to the absorbance at 450 nm. The parameter $A_{281\text{nm}} - A_{450\text{nm}}$ allowed to monitor the denaturation even in the presence of slight turbidity. After denaturation of StP at 50 °C for 20 min in the absence of phosphate (and NaCl), StP was inactive, but the major protein fraction was soluble. The denatured protein was analyzed by gel filtration chromatography. Its molecular mass appeared to be greater than 600 kDa, indicating the formation of soluble high molecular mass aggregates before the onset of the actual precipitation. No enzyme activity could be recovered by diluting, the thus denatured StP into 50 mM phosphate buffer, pH 7.0, to give a final protein concentration between 10 to 1,000 μ g/mL, and incubating that mixture for up to 3 h at 4 or 25 °C. Addition of PLP in 10- to 100-fold molar excess over protein had no effect, indicating that the inactivation of StP was irreversible.

To determine whether dissociation of the protein subunits preceded the aggregation, the denaturation of StP was carried out in Tris buffer at 30 °C. Samples were taken after certain incubation times and analyzed by gel-filtration chromatography. Results are presented in Figure 7A–C. They show the native dimer in Figure 7A and reveal the progressive formation of monomers of StP in dependence of the incubation time (Fig. 7B,C). These findings suggests that (1) subunit dissociation occurs as a result of the denaturation of StP, and (2) the protomer of StP is a kinetically significant intermediate of the denaturation pathway, at least at 30 °C. The stability of the dissociated subunits of StP appears to be quite small. After a 12 h long incubation time at 30 °C, the analysis by gel filtration revealed the absence of the dimeric and monomeric association states, indicating that the aggregation had been

Fig. 6. Thermal denaturation of StP and effect of phosphate on stability, as revealed by measuring the irreversible inactivation of StP, the loss of tertiary structure by near-UV CD, and the increase in absorbance ($A_{281\text{nm}} - A_{450\text{nm}}$). Incubations were carried out in 50 mM Tris buffer, pH 6.9, in the presence of 200 mM P_i and 350 mM NaCl using a protein concentration of 50 μ g/mL (200 μ g/mL for CD, 1 mg/mL for absorbance). Symbols: enzyme activity (full squares); $A_{281\text{nm}} - A_{450\text{nm}}$ (open squares); molar ellipticity at 293 nm in the presence (full circles) and absence of 350 mM NaCl (open circles). Results are normalized by using the initial values at 20 °C (enzyme activity, molar ellipticity at 293 nm) or the value at 60 °C ($A_{281\text{nm}} - A_{450\text{nm}}$) as the basis, and lines are drawn to indicate the trend of the data.

complete (not shown). Under the conditions above, the release into solution of the cofactor was clearly detectable after an incubation time of 30 min and complete after 12 h. By using the same methods of analysis but incubation of StP at 50 °C, it was impossible to observe the formation of a monomeric intermediate, which finding probably reflects the very small life time of the dissociated protein subunit at this high temperature.

Phosphate had a dramatic effect on the thermal denaturation of StP. When 5 mM P_i were added to a Tris-buffered solution of StP, no subunit dissociation and no release of PLP took place during a 24 h long incubation time at 30 °C. In the presence of 200 mM P_i , aggregation of the enzyme occurred at $\approx$ 66 °C, compared with $\approx$ 52 °C when no P_i was present. The temperature at which aggregation of StP became visible by absorbance spectroscopy increased with increasing phosphate concentration, from 63 °C at 20 mM P_i to 70 °C at 500 mM P_i . In the presence of 0.35 M NaCl, however, the temperature of precipitation of StP was downshifted by $\sim$ 10 °C. At 60 °C and greater, it was not possible to distinguish between the release of PLP and the formation of turbidity. To determine whether dissociation of PLP preceded the aggregation at 60–70 °C, StP was denatured in this temperature range until more than 95% of the protein had precipitated. The precipitate was removed by centrifugation, and the amount of PLP in the supernatant and in the precipitate was determined. PLP had accumulated quantitatively in the supernatant, indicating that release of the cofactor occurs during denaturation of StP at all temperatures.

Binding of oxyanions inhibits melting of secondary structure of StP

Changes in the molar ellipticity (Θ) at 222 nm were used to monitor modifications at the level of the secondary structure content of

Fig. 7. Subunit dissociation occurs as a result of the denaturation of StP. The incubation of StP was in 50 mM Tris buffer, pH 6.9, without P_i and at 30 °C. Samples taken at (A) zero time, and (B) after 30 min, and (C) 60 min long incubation times were analyzed by gel filtration chromatography to reveal the association state of the protein. The elution volumes corresponding to the phosphorylase dimer (d) and monomer (m) are indicated by arrows.

StP brought about by thermal denaturation in the range 20 to 80 °C. In the presence of 0.5 mM P_i , structural perturbations occurred already at around 30 °C (Fig. 8). In contrast, with 200 mM P_i being added, there was essentially no change in the value of Θ up to ~63 °C. At temperatures greater than 63 °C, however, the molar ellipticity changed dramatically (Fig. 8). This finding likely reflects the melting of α -helical secondary structure in StP, which, in turn, appeared to be correlated with an increase in the relative content of unordered structure (not shown). When 0.35 M NaCl was added to a 200 mM phosphate buffer, a decrease by as much as 11 °C (from ~63 to ~52 °C) was observed for the temperature at which significant changes in structure of StP began to take

place. The results from far-ultraviolet (UV) CD spectroscopy were fully corroborated by recording near-UV CD spectra between 250 and 320 nm for the same temperature range. Using the absorbance maxima at 273, 285, and 293 nm as characteristic probes of conformational changes in StP, thermal denaturation in 200 mM P_i occurred between 61 and 64 °C that in 200 mM P_i and 0.35 M NaCl at ~53 °C (cf. Fig. 6).

Thermal denaturation pathway of StP revealed by FTIR

Structural changes in StP induced on increasing the temperature from 30 to 70 °C have been monitored by using the deconvoluted spectra recorded at each temperature, and by using the difference spectra calculated from two original spectra recorded in a temperature interval of 5 °C. Data have been obtained in the presence of 2 mM P_i , 50 mM P_i , and 100 mM P_i , and Figure 9 shows as an example the deconvoluted spectra of StP in the presence of 50 mM P_i . The results clearly reveal the occurrence of loss of secondary structure and protein aggregation as a result of thermal denaturation. Typically, the deconvoluted absorbance spectrum recorded at the temperature at which maximum denaturation took place (which was dependent on the phosphate concentration, cf. Fig. 9, 65 °C) showed a broad band centered at 1,644.8 cm^{-1} due to unordered structures and two strong bands at 1,616 and 1,684.4 cm^{-1} due to protein intermolecular interactions (aggregation) brought on by thermal denaturation (Jackson & Mantsch, 1991, 1992; D'Auria et al., 1997). The denaturation of the protein induced also a further $^1\text{H}/^2\text{H}$ exchange as shown by the decrease of the amide II band intensity (1,546.4 cm^{-1}). For StP dissolved in 2 mM P_i , the onset of denaturation and aggregation was observable by using the difference-spectrum technique and occurred at around 35 °C. When using 50 and 100 mM P_i , tiny negative bands close to 1,655 cm^{-1} were detected in the difference spectra between 35 and 50 °C, indicating minor, presumably reversible modifications of the secondary structure of StP in this temperature range (Fig. 10A). In the presence of 50 and 100 mM P_i , denaturation and aggregation of StP started at 50–55 °C (Fig. 10B) and 55–60 °C (Fig. 10C), respectively. Maximum denaturation was observed at 45–50, 60–65, and 65–70 °C for experiments carried out in 2, 50, and 100 mM P_i , respectively (Fig. 10). The analysis by FTIR reveals melting points

Fig. 8. Melting of secondary structure of StP as a result of thermal denaturation. The experiments were carried out with a constant protein concentration of 200 $\mu\text{g}/\text{mL}$. Symbols: 0.5 mM P_i (full squares); 100 mM P_i (open circles); 200 mM P_i (open squares); 200 mM P_i and 350 mM NaCl (full circles). Results are normalized by using the initial values at 20 °C as the basis. Lines are drawn to indicate the trend of the data.

Fig. 9. Deconvoluted spectra of StP in 50 mM phosphate buffer, p^2H 6.8, as a function of the temperature. Spectra are displayed at 5 °C intervals.

of StP, which are slightly (by ≈ 5 °C) higher than those obtained by using CD to monitor the thermal denaturation. That finding is presumably due to a weak stabilization of the enzyme in 2H_2O , related to the larger hydrophobic effect in 2H_2O than H_2O . Heavy water and lyotropic anions have been shown to bring about extra stability of a halophilic malate dehydrogenase by a combined salting-out effect (Bonneté et al., 1994), for example.

The increase in temperature led to other changes in the infrared spectrum as, for instance, amide I' band shift and broadening (Fernandez-Ballester et al., 1992), factors that can be used to monitor the thermal denaturation of a protein. The thermal denaturation of StP was followed by monitoring the amide I' bandwidth, calculated at 3/4 of amide I' band height ($W_{3/4H}$), as a function of the temperature (Fernandez-Ballester et al., 1992; Lippe et al., 1998) (Fig. 11). The thermal stability of StP increased markedly with the increase of P_i concentration, with T_m being estimated to be at ≈ 52 , 64, and 67.5 °C in 2, 50, and 100 mM P_i , respectively. These T_m values agree with the melting points determined from the difference spectra.

Mechanism of thermal denaturation of phosphorylase and role of phosphorylase/oxyanion interactions for stability

Thermal denaturation of StP occurs by a stepwise mechanism (Scheme 1) that involves in that order, release of cofactor and dissociation of subunits; melting of the secondary structure of the

apo-protomers to an extent that is dependent on the incubation temperature; intermolecular association of partially unfolded apo-protomers to give soluble and insoluble aggregates. The dissociation of the cofactor and the formation of protein monomers may

Scheme 1. Denaturation pathway of StP and stabilization by phosphate-ligand binding. The enzyme-bound PLP and phosphate are indicated. N and D are the native protomer and the reversibly denatured protomer, respectively. N' is used to indicate the structural modifications in StP induced by phosphate binding to the oxyanion site; K_{dPi} (≈ 16 mM) is the dissociation constant of this binary complex. U is used to summarize the protomers that show a temperature-dependent degree of unfolding. At 50 °C or greater, inactivation of oxyanion-liganded StP occurs via a reversibly inactivated, dimeric intermediate, as indicated. With the methods used, U is not detectable at 50 °C or greater and therefore shown in brackets. Formation of soluble/insoluble aggregates with a molecular mass of ≥ 600 kDa is indicated by a suffix. Also shown are the temperatures or temperature ranges of the structural transitions in StP. Depending on stabilizing (oxyanions) and destabilizing (e.g., Cl^- , Tris) medium factors, the melting temperature of StP can vary by as much as 17 °C between ≈ 53 and ≈ 70 °C.

Fig. 10. Effect of phosphate on temperature-induced structural modifications, melting of secondary structure and aggregation of StP, using difference spectra between two original absorbance spectra of the enzyme recorded at different temperatures. (A–C) The spectra of StP in 2, 50, and 100 mM P_i . Each difference spectrum is the result of the difference between two original absorbance spectra recorded at the temperatures reported on the left of each difference spectrum (e.g., 25–20 °C). In the 1,700–1,600 cm^{-1} region, negative and positive bands represent the loss of secondary structure and protein aggregation, respectively, which occurred in the sample at higher temperature. The negative band close to 1,545 cm^{-1} is due to further $^1\text{H}/^2\text{H}$ exchange that occurred in the sample at higher temperature.

occur in a single cooperative step or in two steps, which with the methods used, are not well resolved kinetically. Because the promoters of StP are conformationally unstable and prone to aggregation, the monomeric intermediate escapes detection during denaturation of StP at extreme temperatures such as 50 °C or greater.

Fig. 11. Stabilization of StP against thermal denaturation by oxyanion-ligand binding. The thermal denaturation was followed by monitoring the amide I' bandwidth calculated at 3/4 of amide I' band height as a function of temperature. Lines a, b, and c refer to StP (in $^2\text{H}_2\text{O}$) in the presence of 2, 50, and 100 mM P_i , respectively.

Therefore, denaturation of the phosphorylase dimer and the ensuing aggregation appear to take place cooperatively in this temperature range. However, it must be recalled that the monomer presents a kinetically significant intermediate of the denaturation pathway of StP at 30 °C.

By binding to a moderate-affinity oxyanion site, which is different from the phosphate binding site in the active site of StP, phosphate and sulfate induce formation of a more “compact” protein structure of this enzyme (Scheme 1). Interactions between StP and oxyanions are manifested by (1) an increase in the relative content of α -helical secondary structure and (2) a decrease in $^1\text{H}/^2\text{H}$ exchange, compared with the unliganded StP, and indirectly affect the accessibility to solvent of the active-site cofactor. The apparent “conformational tightening” of StP on binding of oxyanions prevents the dissociation of the cofactor and the subunits from taking place. Thereby, it appears to specifically increase the stability of the protein dimer, and we would like to speculate that the binding energy resulting from the protein/oxyanion interaction is translated into tightened intersubunit contacts. The thermostability of StP, measured as irreversible inactivation, is dramatically increased as a result of the stabilized dimer structure. Expressed as the temperature difference that is required to observe similar rates of irreversible inactivation of StP liganded with oxyanion and the free enzyme, binding of phosphate or sulfate brings about a stabilization of StP by at least 20 °C. Even though the functional active site of StP liganded with oxyanion is lost in a reversible manner at ~ 50 °C (Scheme 1), the overall protein structure remains intact at this and much higher temperatures up to 70 °C. Partial inactivation of the oxyanion site, reflected by an in-

crease in the concentration of phosphate required for efficient stabilization of StP, appears to occur concurrently with the loss of the active site at ~50–55 °C. Dimer contact regions and the active site come close together in the structure of α -glucan phosphorylases (Johnson, 1992; Watson et al., 1997), and therefore it is tempting to speculate that the oxyanion site of StP could be at or close to the dimer interface. At even more extreme temperatures of 60 to 70 °C, specific binding together with the salting-out effect of these oxyanions are responsible for the conservation of structure of StP.

In conclusion, binding energy derived from interactions between StP and phosphate or sulfate at the oxyanion site is translated into conformational changes that have a dramatic stabilizing effect on the dimer structure of the protein. The result is a shift of the entire pathway of thermal denaturation of the enzyme to a ≈ 20 °C higher value on the temperature scale. However, interactions at the oxyanion site do not seem to alter the pathway as such. Interestingly, upon activation of rabbit muscle glycogen phosphorylase by phosphorylation at Ser14, this protein assumes a conformation that relative to that of the unphosphorylated enzyme shows a much tighter global association of the subunits (Sprang et al., 1988). Therefore, a similar “conformational tightening” seems to be detectable in StP on phosphate binding and glycogen phosphorylase on phosphorylation. However, it remains to be established whether the binding of P_i to the oxyanion site of StP and the ensuing stabilization of the protein structure bear a particular physiological relevance for *Corynebacterium callunae*.

Despite the great number of proteins that bind compounds containing a phosphoryl group (Copley & Barton, 1994), stabilization by phosphate has been shown for relatively few proteins and enzymes thus far, such as, for example, acid phosphatase (Cashikar & Rao, 1996), RNase (Meiering et al., 1991), the apoenzyme of aspartate aminotransferase (Iriarte et al., 1985; Martinez-Liarte et al., 1992), and maltodextrin phosphorylase from *E. coli* (Grieblér et al., 2000). With the exception of maltodextrin phosphorylase for which phosphate prevents the electrostatically driven aggregation of a reversibly inactivated enzyme dimer, stabilization of the three other enzymes was due to binding of phosphate in the active site, and therefore most significant in the case of the enzyme activity. The mechanism by which phosphate increases the stability of StP against denaturation is clearly different from these examples, and the magnitude of the stabilizing effect appears to be without precedent in the literature. Concerning relationships of structure and function in regulated (Johnson et al., 1990; Johnson, 1992; Lin et al., 1996; Rath et al., 1996) and nonregulated (Watson et al., 1997, 1999; O'Reilly et al., 1998) α -glucan phosphorylases, an excellent basis at high resolution is available. The primary structure of StP has recently been determined (R. Grieblér & B. Nidetzky, unpubl. obs.) and reveals a $\approx 40\%$ identity in amino acid sequence to rabbit muscle glycogen phosphorylase and maltodextrin phosphorylase from *E. coli*. Therefore, by using the structural framework of the α -glucan phosphorylase family, the results reported in this paper could have implications for the design of new stabilizing interactions in oligomeric proteins based on the binding of moderate-affinity oxyanion ligands.

Material and methods

Materials

StP from *C. callunae* DSM 20147 was produced and purified to homogeneity as described recently (Weinhäusel et al., 1997). Deu-

terium oxide (99.9% $^2\text{H}_2\text{O}$) was from Sigma (St. Louis, Missouri). Maltodextrin with a dextrin-equivalent of 19.4 was obtained from Agrana (Gmünd, Austria), and soluble starch was from Merck (Darmstadt, Germany). All the other chemicals were of highest purity available from Sigma.

Assays and other measurements

Phosphorylase activity was measured in the direction of phosphorolysis at 30 °C by using a continuous, coupled enzyme assay described recently (Weinhäusel et al., 1997). α -D-Glc 1-P was determined by a discontinuous enzymatic assay (Weinhäusel et al., 1997), or by high-performance anion chromatography (Eis & Nidetzky, 1999). Enzyme-bound PLP was detected qualitatively by absorbance at 330–332 nm. Quantitative PLP analysis was done according to Wada and Snell (1961). The oligomeric structure of phosphorylase was determined by gel filtration at 20 °C using a prepacked Superose 12 HR 10/30 column (Amersham Pharmacia Biotech, Freiburg, Germany) with the fast protein liquid chromatography system. The column was equilibrated with 50 mM phosphate buffer containing 0.2 M NaCl, and elution was carried out at 0.2 mL/min using the same buffer. About 100 μg protein were applied to the column.

Fluorescence measurements

All fluorescence measurements were carried out on a Hitachi Spectrofluorometer F 2000 (Hitachi, Tokyo, Japan) at 30 ± 1 °C in cells of a 1 cm optical path using slit widths of 5 nm for the excitation and emission wavelength. The fluorescence of protein-bound PLP in phosphorylase was recorded between 450–600 nm, following excitation at 330 nm. Quenching by iodide of this fluorescence was determined in the presence or absence of ligands using increasing concentrations of potassium iodide up to 0.8 M, while the total salt concentration was kept constant at 1.0 M using potassium chloride. Each fluorescence measurement was recorded in triplicate to reduce errors, and the data shown represent mean values of these independent readings. The appropriate blank buffer values were subtracted from all fluorescence intensities.

Temperature dependence of phosphorylase activity

The substrate solution (50 mM Tris buffer, pH 6.9) was incubated in Eppendorf tubes at the desired temperature for 30 min. Before the enzyme was added, the temperature was controlled, and values reported varied within ± 1 °C. Twenty milliliters of concentrated enzyme was added to the substrate to give a final protein concentration of 5 $\mu\text{g}/\text{mL}$ in the assay. Incubation was continued for exactly 15 min under gentle mixing in an Eppendorf Thermomixer (model 5436, Eppendorf, Köln, Germany) with instrument settings at $400 \times \text{rpm}$. After 5, 10, and 15 min, samples of 30 μL were taken and diluted immediately into the enzymatic assay for determination of Glc 1-P. Initial velocities were derived from the linear relationship of [Glc 1-P] produced vs. time. Kinetic parameters for the phosphorolysis and synthesis reaction catalyzed by StP were determined from initial velocity measurements at different temperatures by using phosphate or Glc 1-P as the varied substrates (5–500 mM) and soluble starch at a constant concentration of 60 g/L.

Stability of phosphorylase activity

StP was incubated at a protein concentration of 50 $\mu\text{g}/\text{mL}$ in buffers and at temperatures indicated, under otherwise identical conditions as described above. Phosphate was added to the buffer in concentrations indicated. At suitable times (0.5–10 min) and up to ~ 2 h reaction time, 20 μL samples were taken and diluted immediately into the coupled phosphorylase assay. Inactivation rate constants were obtained from plots of $\ln(A_t/A_0)$ vs. incubation time, where A_t is the residual activity at time t , and A_0 is the initial enzyme activity.

Absorbance spectroscopy

This was carried out with a Hitachi double-beam spectrophotometer model UV 3000 equipped with Hitachi temperature controller model SPR-10. The matched reference cuvette contained all components of the sample cuvette (350 μL), except the phosphorylase (1 mg/mL). A temperature step program was carried out in the range of 25 to 70°C, in which the temperature was raised automatically between two measurement temperatures at a constant speed of 1°C/min. The temperature precision was $\pm 0.2^\circ\text{C}$. An absorbance spectrum of the phosphorylase (270–600 nm) was recorded in intervals of 3–5°C, and the holding time at the corresponding temperature was 5 min. The absorbance peaks at 281, 332, and 405 nm, as well as the absorbance valley at 450 nm were used to monitor the denaturation. Thermal denaturation of StP was followed in 50 mM Tris buffer, pH 6.9, containing different concentrations of P_i between 5 and 300 mM. The control contained less than 0.1 mM P_i .

CD spectroscopy

CD spectroscopy was performed at concentrations of StP between 0.2 and 0.5 mg/mL in the presence of 2.0, or 50, or 100 mM sodium phosphate buffer, pH 6.8. A J-710 spectropolarimeter (Jasco, Tokyo, Japan) was used. It was equipped with the Neslab RTE-110 temperature-controlled liquid system (Neslab Instruments, Frankfurt, Germany) and calibrated with a standard solution of (+)-10-camphorsulfonic acid. Sealed cuvettes with a 0.1 and 1.0 cm pathlength (Helma, Müllheim, Germany) were used in the far- and near-UV region, respectively. Photomultiplier high voltage did not exceed 600 V in the spectral regions measured. Each spectrum was averaged five times and smoothed with Spectropolarimeter System Software version 1.00 (Jasco). All measurements were performed under nitrogen flow. Before undergoing CD analyses, all samples were kept at the temperature being studied for 6 min. The results are expressed in terms of residue molar ellipticity (Θ).

FTIR measurements

Buffer exchange and replacement of H_2O by $^2\text{H}_2\text{O}$ were done by using 30K microsep micro-concentrators (Filtron, Northborough, Massachusetts) at 3,000 $\times g$ and 4°C. The reported p²H value corresponds to the pH-meter reading +0.4 (Salomaa et al., 1964). Steps of sixfold concentration and dilution with the final buffer by the same factor were repeated at least 10 times. All samples were concentrated to a final volume of ~ 40 μL (≈ 40 mg/mL) and used for the infrared analysis.

The concentrated protein samples in the final buffers were placed in a thermostated Graseby Specac 20500 cell (Graseby Specac

Ltd., Orpington, UK) fitted with CaF_2 windows and 25 μm spacers. FTIR spectra were recorded by means of a Perkin-Elmer 1760-X Fourier transform infrared spectrometer equipped with a Perkin-Elmer 7500 data station and CDS-3 software for collecting and processing spectra. A deuterated triglycine sulfate detector and a normal Beer–Norton apodization function were used in all experiments. At least 24 h before and during data acquisition, the spectrometer was continuously purged with dry air at a dew point of -40°C . Spectra of buffers and samples were acquired at 2 cm^{-1} resolution under the same scanning and temperature conditions. Typically, 256 scans were averaged for each spectrum obtained at 20°C, while 16 scans were averaged for spectra obtained at higher temperatures. In the thermal-denaturation experiments, the temperature was raised in 5°C steps from 20 to 90°C. Before spectrum acquisition, samples were maintained at the desired temperature for the time necessary for the stabilization of temperature inside the cell (5 min). The deconvoluted spectra were calculated by using the CDS-3 program and in particular using the ENHANCE function, which is analogous to the method developed by Kauppinen et al. (1981). The deconvoluted parameters for the amide I band were set with the half-bandwidth at 18 cm^{-1} and a resolution enhancement factor of 2.5.

Acknowledgments

Financial support was from projects of the Austrian Science Fund (FWF; grants P09720-MOB and P11898-MOB to B.N.), and a travel grant from the programme for technical-scientific collaboration between Austria and Italy (grant No. 21, to B.N. and S.D'A.). Dr. A. Weinhäusel and Mr. B. Müller-Fembeck are thanked for their contributions in an early stage of this work.

References

- Arrondo JLR, Muga A, Castresana J, Goni FM. 1992. Quantitative studies of the structure of proteins in solutions by Fourier transform infrared spectroscopy. *Prog Biophys Mol Biol* 59:23–56.
- Baldwin RL. 1996. How Hofmeister ion interactions affect protein stability. *Biophys J* 71:2056–2063.
- Banuelos S, Arrondo JLR, Goni FM, Pitag G. 1995. Surface-core relationships in human low density lipoprotein as studied by infrared spectroscopy. *J Biol Chem* 270:9192–9196.
- Barford D, Hu S-H, Johnson LN. 1991. Structural mechanism for glycogen phosphorylase control by phosphorylation and AMP. *J Mol Biol* 218:233–260.
- Barford D, Johnson LN. 1989. The allosteric transition of glycogen phosphorylase. *Nature* 340:609–616.
- Bartl F, Palm D, Schinzel R, Zundel G. 1999. Proton relay system in the active site of maltodextrinphosphorylase via hydrogen bonds with large proton polarizability: An FT-IR difference spectroscopy study. *Eur Biophys J* 28:200–207.
- Becker S, Palm D, Schinzel R. 1994. Dissecting differential binding in the forward and reverse reaction of *Escherichia coli* maltodextrin phosphorylase using 2-deoxyglucosyl substrates. *J Biol Chem* 269:2485–2490.
- Bonneté F, Madern D, Zaccari G. 1994. Stability against denaturation mechanisms in halophilic malate dehydrogenase “adapt” to solvent conditions. *J Mol Biol* 244:436–447.
- Byler DM, Susi H. 1986. Examination of the secondary structure of proteins by deconvoluted FTIR spectra. *Biopolymers* 25:469–487.
- Cashikar AG, Rao NM. 1996. Unfolding pathway in red kidney bean acid phosphatase is dependent on ligand binding. *J Biol Chem* 271:4741–4746.
- Chirgadze YN, Fedorow OW, Trushina NP. 1975. Estimation of amino acid residue side-chain absorption in the infrared spectra of protein solutions in heavy water. *Biopolymers* 14:679–694.
- Copley RR, Barton GJ. 1994. A structural analysis of phosphate and sulfate binding sites in proteins. *J Mol Biol* 242:321–329.
- Cortijo M, Steinberg IZ, Shaltiel S. 1971. Fluorescence of glycogen phosphorylase b. *J Biol Chem* 246:933–938.
- D'Auria S, Barone R, Rossi M, Nucci R, Barone G, Fessas D, Bertoli E, Tanfani F. 1997. Effects of temperature and SDS on the structure of β -glycosidase

- from the thermophilic archaeon *Sulfolobus solfataricus*. *Biochem J* 323: 833–840.
- Eis C, Nidetzky B. 1999. Characterization of trehalose phosphorylase from *Schizophyllum commune*. *Biochem J* 341:385–393.
- Engers HD, Shechosky S, Madsen NB. 1970. Kinetic mechanism of phosphorylase *a*. I. Initial velocity studies. *Can J Biochem* 48:746–754.
- Fernandez-Ballester G, Castresana J, Arrondo, JLR, Ferragut JA, Gonzales-Ros JM. 1992. Protein stability and interaction of the nicotinic acetylcholine receptor with cholinergic ligands studied by Fourier-transform infrared spectroscopy. *Biochem J* 288:421–426.
- Gold A, Johnson RM, Tseng JK. 1970. Kinetic mechanism of rabbit muscle glycogen phosphorylase *a*. *J Biol Chem* 245:2564–2572.
- Goldsmith EJ, Sprang SR, Hamlin R, Xuong N-H, Fletterick RJ. 1989. Domain separation in the activation of glycogen phosphorylase *a*. *Science* 245:528–532.
- Grießler R, D'Auria S, Schinzel R, Tanfani F, Nidetzky B. 2000. Mechanism of thermal denaturation of maltodextrin phosphorylase from *Escherichia coli*. *Biochem J* 346:255–263.
- Grießler R, Müller-Fembeck B, D'Auria S, La Cara F, Nidetzky B. 1998. Stability and stabilization of α -1,4-D-glucan phosphorylases. In: Ballesteros A, Plou FJ, Iborra J, Halling P, eds. *Progress in biotechnology—Stability and stabilization of biocatalysts*. Amsterdam, The Netherlands: Elsevier. pp 83–88.
- Honikel KO, Madsen NB. 1973. Fluorescence quenching, a tool for probing conformational changes in glycogen phosphorylase. *Can J Biochem* 51: 344–356.
- Iriarte A, Kraft K, Martinez-Carrion M. 1985. The separate effects of coenzyme components may not be additive. *J Biol Chem* 260:7457–7463.
- Jackson M, Mantsch HH. 1991. Beware of proteins in DMSO. *Biochim Biophys Acta* 1078:231–235.
- Jackson M, Mantsch HH. 1992. Halogenated alcohols as solvents for proteins: FTIR spectroscopic studies. *Biochim Biophys Acta* 1118:139–143.
- Johnson LN. 1992. Glycogen phosphorylase: Control by phosphorylation and allosteric effectors. *FASEB J* 6:2274–2282.
- Johnson LN, Acharya KR, Jordan MD, McLaughlin PJM. 1990. Refined crystal structure of the phosphorylase-heptulose 2-phosphate-oligosaccharide-AMP complex. *J Mol Biol* 211:645–661.
- Johnson LN, Snape P, Martin JL, Acharya KR, Barford D, Oikonomakos NG. 1993. Crystallographic binding studies on the allosteric inhibitor glucose-6-phosphate to T state glycogen phosphorylase. *J Mol Biol* 232:253–267.
- Kauppinen JK, Moffat DJ, Mantsch HH, Cameron DG. 1981. Fourier self-deconvolution: A method for resolving intrinsically overlapped bands. *Appl Spectrosc* 35:271–276.
- Lin K, Rath VL, Dai SC, Fletterick RJ, Hwang PK. 1996. A protein phosphorylation switch at the conserved allosteric site in glycogen phosphorylase. *Science* 273:1539–1541.
- Lippe G, Tanfani F, Di Pancrazio F, Contessi S, Bertoli E, Dabbeni-Sala F. 1998. Effect of inhibitor binding to β subunits of F_1F_0 ATPase on enzyme thermostability: A kinetic and FT-IR spectroscopic analysis. *FEBS Lett* 432:128–132.
- Madsen NB, Withers SG. 1986. Glycogen phosphorylase. In: Dolphin D, Poulson R, Avramovic O, eds. *Coenzymes and cofactors, Vol. I, part B—Vitamin B₆ pyridoxal phosphate*. New York: J. Wiley & Sons. pp 355–389.
- Martinez-Liarte JH, Iriarte A, Martinez-Carrion M. 1992. Inorganic phosphate binding and electrostatic effects in the active center of aspartate aminotransferase apoenzyme. *Biochemistry* 31:2712–2719.
- Meiering EM, Bycroft M, Fersht AR. 1991. Characterization of phosphate binding in the active site of barnase by site-directed mutagenesis. *Biochemistry* 30:11348–11356.
- Muga A, Arrondo JLR, Bellon T, Sancho J, Bernabeu C. 1993. Structural and functional studies on the interaction of sodium dodecyl sulfate with β -galactosidase. *Arch Biochem Biophys* 300:451–457.
- Newgard CB, Hwang PK, Fletterick RJ. 1989. The family of glycogen phosphorylases: Structure and function. *CRC Crit Rev Biochem Mol Biol* 24:69–99.
- O'Reilly M, Watson KA, Schinzel R, Palm D, Johnson LN. 1998. Oligosaccharide substrate binding to *E. coli* maltodextrin phosphorylase. *Nat Struct Biol* 4:405–412.
- O'Reilly M, Watson KA, Johnson LN. 1999. The crystal structure of the *E. coli* maltodextrin phosphorylase–acarbose complex. *Biochemistry* 38:5337–5345.
- Osborne HB, Nabadryk-Viala E. 1982. Infrared measurement of peptide hydrogen exchange in rhodopsin. *Methods Enzymol* 88:676–680.
- Palm D, Klein HW, Schinzel R, Buehner M, Helmreich EJM. 1990. The role of pyridoxal 5'-phosphate in glycogen phosphorylase catalysis. *Biochemistry* 29:1099–1107.
- Rath V, Lin K, Hwang PK, Fletterick RJ. 1996. The evolution of an allosteric site in phosphorylase. *Structure* 4:463–473.
- Salomaa P, Shaleger LL, Long FA. 1964. Solvent deuterium isotope effects on acid-base equilibria. *J Am Chem Soc* 86:1–7.
- Schinzel R, Drueckes P. 1991. The phosphate recognition site of *Escherichia coli* maltodextrin phosphorylase. *FEBS Lett* 286:125–128.
- Shaltiel S, Hedrick JL, Pocker A, Fischer EH. 1969. Reconstitution of apophosphorylase with pyridoxal 5'-phosphate analogs. *Biochemistry* 8:5189–5196.
- Shimomura S, Emman K, Fukui T. 1980. The role of pyridoxal 5'-phosphate in plant phosphorylase. *J Biochem* 87:1043–1052.
- Sprang SR, Acharya KR, Goldsmith EJ, Stuart DI, Varvill K, Fletterick RJ, Madsen NB, Johnson LN. 1988. Structural changes in glycogen phosphorylase induced by phosphorylation. *Nature* 336:215–221.
- Sprang SR, Madsen NB, Withers SG. 1992. Multiple phosphate positions in the catalytic site of glycogen phosphorylase: Structure of the pyridoxal-5'-phosphate coenzyme-substrate analog. *Protein Sci* 1:1100–1111.
- Sprang SR, Withers SG, Goldsmith EJ, Fletterick RJ, Madsen NB. 1991. Structural basis for the activation of glycogen phosphorylase *b* by adenosine monophosphate. *Science* 254:1367–1371.
- Stirtan WG, Withers SG. 1996. Phosphonate and α -fluorophosphonate analogue probes of the ionization state of pyridoxal 5'-phosphate (PLP) in glycogen phosphorylase. *Biochemistry* 35:15057–15064.
- Wada H, Snell EE. 1961. The enzymatic oxidation of pyridoxine and pyridoxamine phosphates. *J Biol Chem* 236:2089–2095.
- Watson KA, Schinzel R, Palm D, Johnson LN. 1997. The crystal structure of *Escherichia coli* maltodextrin phosphorylase provides an explanation for the activity without control in this basic archetype of a phosphorylase. *EMBO J* 16:1–14.
- Watson KA, McCleverty C, Geremia S, Cottaz S, Driguez H, Johnson LN. 1999. Phosphorylase recognition and phosphorolysis of its oligosaccharide substrate: Answers to a long outstanding question. *EMBO J* 18:4619–4632.
- Weinhäusel A, Grießler R, Krebs A, Zipper P, Haltrich D, Kulbe KD, Nidetzky B. 1997. α -1,4-D-glucan phosphorylase of gram-positive *Corynebacterium callunae*: Isolation, biochemical properties and molecular shape of the enzyme from solution X-ray scattering. *Biochem J* 326:773–783.