Mechanism of thermal denaturation of maltodextrin phosphorylase from *Escherichia coli*

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Maltodextrin phosphorylase from *Escherichia coli* (MalP) is a dimeric protein in which each ≈ 90 -kDa subunit contains active-site pyridoxal 5'-phosphate. To unravel factors contributing to the stability of MalP, thermal denaturations of wild-type MalP and a thermostable active-site mutant (Asn-133 $\rightarrow$ Ala) were compared by monitoring enzyme activity, cofactor dissociation, secondary structure content and aggregation. Small structural transitions of MalP are shown by Fourier-transform infrared spectroscopy to take place at ≈ 45 °C. They are manifested by slight increases in unordered structure and $^1\text{H}/^2\text{H}$ exchange, and reflect reversible inactivation of MalP. Aggregation of the MalP dimer is triggered by these conformational changes and starts at ≈ 45 °C without prior release into solution of pyridoxal 5'-phosphate. It is driven by electrostatic rather than hydrophobic interactions between MalP dimers, and leads to irreversible inactivation of the enzyme. Aggregation is inhibited efficiently and specifically by oxyanions such as phosphate, and AMP

which therefore, stabilize MalP against the irreversible denaturation step at 45 °C. Melting of the secondary structure in soluble and aggregated MalP takes place at much higher temperatures of approx. 58 and 67 °C, respectively. Replacement of Asn-133 by Ala does not change the mechanism of thermal denaturation, but leads to a shift of the entire pathway to a ≈ 15 °C higher value on the temperature scale. Apart from greater stability, the Asn-133 $\rightarrow$ Ala mutant shows a 2-fold smaller turnover number and a 4.6-fold smaller energy of activation than wild-type MalP, probably indicating that the site-specific replacement of Asn-133 brings about a greater rigidity of the active-site environment of the enzyme. A structure-based model is proposed which explains the stabilizing interaction between MalP and oxyanions, or AMP.

Key words: glucan phosphorylase, oxyanion site, phosphate, stabilization.

INTRODUCTION

Maltodextrin phosphorylase (MalP) is a cytosolic enzyme component of the maltodextrin-metabolizing protein machinery in *Escherichia coli* [1]. It is a dimer composed of two identical subunits of 796 amino acids [2]. Each protomer has bound one molecule of pyridoxal 5'-phosphate (PLP) as the active-site cofactor which is essential for activity. The enzyme catalyses the reversible phosphorolysis of maltopentaose or longer malto-oligosaccharides according to the equation:

where DP is the degree of polymerization of the maltodextrin.

The structure of MalP unliganded with α -glucan substrate has been solved at high resolution [3]. It has established details of the subunit–subunit interactions in MalP and on comparison with phosphorylated and non-phosphorylated rabbit muscle glycogen phosphorylase, how these interactions relate to enzyme activity of MalP in the absence of activators. The structures of binary complexes of MalP with maltotetraose [4] and acarbose [5], and a very recent structure of the ternary complex of MalP with phosphate and a non-phosphorolysable thio-oligosaccharide substrate analogue [6] provide a detailed picture on substrate recognition by MalP and the catalytic mechanism of α -glucan phosphorylase.

Dimeric proteins are thought to be on average 4–13 kcal/mol more stable than the corresponding monomers [7]. In their denaturation pathways, loss of catalytic activity usually belongs to the steps that occur early, in which either the oligomeric structure is lost because of subunit dissociation, or the active-site integrity is destroyed by other often small conformational changes [e.g. 8–12]. For α -glucan phosphorylases, the interactions at the dimer interface are quite well known [3,13–18]. However, the relationship between enzyme activity, conformational stability and apparent strength of the dimer contacts has not been investigated in detail so far. By studying effects of guanidinium hydrochloride on enzyme activity and structural integrity of rabbit muscle glycogen phosphorylase, Price and Stevens [19] concluded that this phosphorylase is much more sensitive to chemical denaturation than a number of other oligomeric proteins. A marked conformational flexibility of glycogen phosphorylase which seems to be required to achieve the complex regulatory properties of this enzyme [13–18], has been hypothesized to bring about the relatively low stability of the phosphorylase [19]. Large differences have recently been observed among individual α -glucan phosphorylases from bacteria, plant and mammals regarding the thermostability of enzyme activity [20]. We would like to understand what features of structure are important elements of stability in the α -glucan phosphorylase family. A prerequisite to advance knowledge in this area is, however, that denaturation pathways of individual phosphorylases be known precisely, which is not the case for MalP and other α -glucan phosphorylases.

Abbreviations used: amide I', amide I band in $^2\text{H}_2\text{O}$ medium; FT-IR, Fourier-transform infrared spectroscopy; Glc 1-P, α -D-glucose 1-phosphate; MalP, maltodextrin phosphorylase from *Escherichia coli*; PLP, pyridoxal 5'-phosphate; P_i , inorganic phosphate; T_m , melting temperature.

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The present paper is concerned with a characterization of the denaturation mechanisms of MalP and an active-site mutant of MalP (Asn-133 → Ala) [3] at elevated temperatures. The mutant has recently been found to be much more thermostable than wild-type MalP (R. Schinzel, unpublished work) but so far, the reason for its greater stability has remained unclear. It seemed interesting, therefore, to compare MalP and the mutant phosphorylase in thermal denaturation studies. Asn-133 does not directly interact with the glucan substrate of phosphorylase, but shows interactions with amino acids that are involved in substrate binding [4–6]. (Note that the numeration of mammalian glycogen phosphorylase is used here for reason of better comparison with work published on MalP structure [3–6].)

The results show that the denaturation pathway of MalP is sequential and includes reversible inactivation, probably brought about by disturbances of the active-site integrity, destabilization of the protein by exposure of aggregation-prone surface, and irreversible aggregation of the PLP-containing dimer triggered by ionic intermolecular interactions. The structural core of MalP has a stable secondary structure which melts about 18 °C above the temperature at which destabilization of the dimer starts. For the thermostable Asn-133 → Ala, the entire denaturation pathway is shifted to a ≈ 15 °C higher value on the temperature scale but there is no change in mechanism as such. Comparison of stability and activity of wild-type MalP and the Asp-133 → Ala mutant shows that the greater stability of enzyme activity is correlated with a decreased turnover number. A model based on MalP 3-D structure [3,4] is used to interpret the stabilization of phosphorylase activity by oxyanions such as phosphate, and AMP.

EXPERIMENTAL

Materials

Recombinant MalP and the Asn-133 → Ala mutant [3] were produced and purified to homogeneity as described [21]. Deuterium oxide (99.9% $^2\text{H}_2\text{O}$) was purchased from Aldrich. Maltodextrin with a dextrin equivalent of 19.4 was from Agrana (Gmünd, Austria). All the other chemicals were of the purest quality and obtained from Sigma.

Assays and other measurements

Phosphorylase activity was measured in the direction of phosphorylation at 30 °C by using a continuous, coupled enzyme assay described recently [22]. α -D-glucose 1-phosphate (Glc 1-P) was determined by a discontinuous enzymatic assay [22], or by HP anion chromatography on a Dionex HPLC system, model DX-120 (Dionex, Sunnyvale, CA, U.S.A.) with conductivity detection. PLP was measured by spectrophotometric (332 nm) or spectrofluorimetric (exc. 332 nm; em. 550 nm) analyses [22]. Light scattering was measured on a Hitachi F2000 spectrofluorometer at 25 °C with excitation and emission wavelengths at 550 nm. Quantitative PLP analysis was done according to Wada and Snell [23]. The possible release of ammonia from MalP by deamidation at elevated temperatures was determined enzymatically, by using the NADH-dependent reductive amination of oxoglutarate catalysed by glutamate dehydrogenase from beef liver and measuring the consumption of NADH by absorbance (340 nm) or fluorescence (exc. 360 nm, em. 450 nm). Kinetic parameters of MalP and the Asn-133 → Ala mutant were determined at 30 °C in phosphorylation by using maltodextrin as the α -glucan substrate and measuring initial velocities with the discontinuous enzyme assay [22]. Parameter estimates were

obtained from nonlinear fits of the data to a one-substrate Michaelis–Menten equation with substrate inhibition, using the least-squares method (Sigmaplot Vers. 5, Jandel).

Temperature dependence of phosphorylase activity

One ml of substrate solution containing maltodextrin (60 mg/ml) and phosphate (10–300 mM) dissolved in 50 mM Tris buffer, pH 6.9, was pre-equilibrated in Eppendorf tubes at the temperature of the experiment (30–70 °C) for 30 min. The temperature in the tubes was controlled before enzyme was added, and values reported varied within ± 1 °C. Twenty μl of a concentrated enzyme preparation was added to the tubes to give a 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) with instrument settings at 400 rev./min. 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 versus time. The turnover numbers (k_{cat}) were calculated from the hyperbolic dependence of the initial velocities on the phosphate concentration by using a nonlinear fitting procedure. The energy of activation (E_a) for k_{cat} was calculated from the slope of a plot of $\ln k_{\text{cat}}$ versus $1/T$ (K), using the relationship slope = E_a/R , where R is the gas constant ($8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). The $\Delta H^\ddagger$ values for phosphorylation at a given temperature were calculated according to $\Delta H^\ddagger = E_a - RT$. The activation free energies ($\Delta G^\ddagger$) of the reactions catalysed by MalP and Asn-133 → Ala mutant were calculated according to $\Delta G^\ddagger = -RT \ln(k_{\text{cat}} h/kT)$, where h ($6.6256 \times 10^{-34} \text{ J} \cdot \text{s}$) is Planck's constant and k ($1.3805 \times 10^{23} \text{ J/K}$) is the Boltzmann constant. The relationship $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$ was used to calculate $\Delta S^\ddagger$.

Stability of phosphorylase activity

MalP and the Asn-133 → Ala mutant were incubated at a protein concentration of 50 $\mu\text{g}/\text{ml}$ in 50 mM Tris buffer, pH 6.9, at 45 and 60 °C, respectively, under otherwise identical conditions as described above. In suitable time intervals up to approx. 2 h incubation time, 20- μl samples were taken and diluted immediately into the coupled phosphorylase assay. The remaining enzyme activity after a certain period of elapsed time reflected in all cases irreversible enzyme inactivation since no recovery of phosphorylase activity was observed on incubating partially inactivated enzyme at room temperature for up to 3 h. Inactivation rate constants were obtained from linear plots of $\ln(A_t/A_0)$ versus time, where A_t is the residual activity at time t , and A_0 is the initial enzyme activity.

Spectrophotometric analysis of temperature-dependent stability of MalP

This was carried out with a Hitachi double-beam spectrophotometer model UV 3000 equipped with a Hitachi temperature-controller model SPR-10. To determine conformational changes of MalP induced by temperature, a constant protein concentration of 0.4 mg/ml, dissolved in 50 mM Tris buffer, pH 6.9, was used in all experiments. Starting from 25 °C, the temperature was raised in steps of 5 °C with a temperature increase of 1 °C/min. The holding time at each temperature was 5 min, and a full absorbance spectrum from 260 to 450 nm was recorded at each temperature. The absorbance peaks at 281 and 332 nm as well as the absorbance valley at 450 nm were used to monitor the denaturation. Quartz cuvettes with a

sample volume of 300 μ l were used, and the reference cuvette contained buffer.

Fourier-transform infrared spectroscopy (FT-IR)

Typically, 1.5 mg of MalP in 100 mM phosphate buffer, pH 6.8, was centrifuged in a 30 K microstep micro-concentrator (Filtron, Northborough, MA, U.S.A.) at 3000 *g* and 4 °C to a volume of approx. 50 μ l. Then 300 μ l of phosphate buffer (2, 50 or 100 mM) in $^2\text{H}_2\text{O}$, p ^2H 6.8, was added and the sample concentrated again. This procedure was repeated 10 times, and finally all samples were concentrated to approx. 40 μ l 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, Kent, U.K.) fitted with CaF_2 windows and 25 μ m spacers. FT-IR spectra were recorded by means of a Perkin–Elmer 1760-x Fourier-transform infrared spectrometer using a deuterated triglycine sulphate detector and a normal Beer–Norton apodization function. 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 steps of 5 °C 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 (6 min). Precipitated MalP was placed between the two CaF_2 windows together with a few drops of 2 mM phosphate buffer, p ^2H 6.8, and then the 20 500 cell was assembled and used for the infrared analysis. 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.

RESULTS

Kinetic parameters of MalP and the Asn-133 $\rightarrow$ Ala mutant

Apart from a 2-fold difference in turnover number and a small difference in K_m^{glucan} , kinetic parameters for phosphorolysis of maltodextrin at 30 °C are identical within experimental error for wild-type MalP and the Asn-133 $\rightarrow$ Ala mutant (Table 1). Apparent binding of a phosphate is strongly dependent on which buffer is used for the experiment. It is approx. 20-fold weaker in phosphate buffer, compared with 50 mM Tris or triethanolamine buffers in which a value of $K_m^{\text{phosphate}}$ of approx. 1 mM is obtained for both the wild-type enzyme and the mutant.

Table 1 Kinetic parameters in direction of maltodextrin phosphorolysis

Parameter ^a	MalP	Asn-133 $\rightarrow$ Ala
k_{cat} (s^{-1}) ^b	25 ± 3	13 ± 2
K_m^{glucan} (g/l) ^c	2.8 ± 0.3	4.1 ± 0.4
$K_m^{\text{phosphate}}$ (mM) ^d	27 ± 2 (≈ 1) ^b	29 ± 1 (≈ 1) ^e

^a Determined at 30 °C in phosphate buffer, pH 6.9; ^b based on a molecular mass of MalP subunit of 90 kDa; ^c determined at 300 mM phosphate; ^d determined at 50 g/l maltodextrin; ^e in parentheses, value determined in 50 mM triethanolamine or 50 mM Tris buffers, pH 6.9.

Figure 1 Stabilization by phosphate of MalP at 45 °C (●) and the Asn-133 $\rightarrow$ Ala mutant at 60 °C (○)

Reactions were carried out in 50 mM Tris buffer, pH 6.9, using 50 μ g/ml enzyme. Half-life times (τ) are calculated from apparent first-order inactivation constants, k_m (min^{-1}), according to $\tau = \ln 2/k_m$. The stabilization of MalP (□) and Asn-133 $\rightarrow$ Ala (■) by AMP at 50 mM and 100 mM is shown.

Stability of MalP and the Asn-133 $\rightarrow$ Ala mutant

Relative to the wild-type enzyme, the Asn-133 $\rightarrow$ Ala mutant shows much greater thermostability: the first-order rate constant for the irreversible inactivation of MalP at 45 °C equals that of the mutant at 60 °C (Figure 1). On incubation of MalP (2–5 mg/ml) at 45 °C for 2 h in the presence of 300 mM phosphate, there was no detectable release of ammonia from the protein into solution. Therefore, deamidation of Asn-133 or other Asn or Gln residues does not contribute to the inactivation of wild-type MalP.

MalP and the mutant phosphorylase are stabilized by phosphate ions. Stabilization depends on the concentration of phosphate (Figure 1) and is quite specific for phosphate. A notable exception is AMP which stabilizes MalP and the mutant as efficiently as phosphate (Figure 1). In contrast, ATP does not stabilize at all. Phosphorylated sugars such as the substrate Glc 1-P or glucose 6-phosphate provide no protection against inactivation of both enzymes. Other oxyanions such as sulphate and arsenate have a more than 10-fold reduced potential for stabilizing MalP and the Asn-133 $\rightarrow$ Ala mutant when compared with phosphate. In summary, these results suggest that stabilization of the enzyme activity depends on specific interactions of MalP or the mutant with phosphate ions or AMP. They argue strongly against an unspecific lyotropic-anion effect of phosphate in conferring extra stability to the enzymes. According to their salting-out potential [24], sulphate and phosphate should have similar effectiveness in stabilizing MalP and Asn-133 $\rightarrow$ Ala, which is not observed.

The dependence on the phosphate concentration of the half-life time of MalP is not simple and appears biphasic (Figure 1). In contrast, a plot of the half-life time at 60 °C versus phosphate concentration is linear for the Asn-133 $\rightarrow$ Ala mutant (Figure 1). The data obtained at low concentrations of phosphate (≤ 0.1 M) allow to estimate that the stabilization by phosphate is approx.

Figure 2 Temperature dependence of the turnover numbers of MalP (●) and Asn-133 → Ala mutant (○)

Initial velocities were measured in phosphorolysis at pH 6.9 (50 mM Tris buffer) with phosphate as the varied substrate and in the presence of saturating concentrations of maltodextrin (60 g/l). The arrows indicate at which temperature absorbance and light-scattering measurements have shown the onset of protein aggregation.

8-fold greater for MalP at 45 °C, compared with that for the mutant at 60 °C.

Three pieces of evidence provide strong experimental support for the suggestion that the stabilization by phosphate does not originate from protein-phosphate interactions in the active site of MalP. (1) Glc 1-P does not stabilize MalP or Asn-133 → Ala against irreversible inactivation. (2) Glc 1-P does not inhibit the phosphate effect on MalP stability. (3) The stabilizing AMP

neither quenches nor affects otherwise the cofactor fluorescence of MalP or of the mutant. In addition, it does not affect the chemical quenching by iodide of the PLP fluorescence of wild type or the mutant (results not shown).

Optimum temperature of catalysis by MalP and the Asn-133 → Ala mutant

The dependence on the temperature of the turnover numbers of MalP and the mutant enzyme has been derived from initial velocity measurements in the range 30–70 °C with phosphate as the varied substrate and using maltodextrin at a constant saturating concentration (60 g/l). The temperature profiles of k_{cat} reveal values of 40 and 55 °C for the optimum temperatures of MalP and the Asn-133 → Ala mutant, respectively (Figure 2). Between 30 and 40 °C there is an approx. 2.9-fold increase in k_{cat} for MalP (Figure 2), corresponding to a free energy of activation for the rate-limiting step of 83.8 kJ/mol. The thermodynamic parameters for the reaction catalysed by MalP have been calculated for $T = 303$ K. They are: $\Delta H^\ddagger$ 81 kJ/mol, $\Delta G^\ddagger$ 333 kJ/mol, $\Delta S^\ddagger$ 832 J·mol⁻¹·K⁻¹. Interestingly, the increase with temperature of k_{cat} for the mutant phosphorylase is significantly smaller than that for the wild-type enzyme (Figure 2). It is about 2.8-fold between 30 and 55 °C. Therefore, the free energy of activation for k_{cat} is 18 kJ/mol. The corresponding thermodynamic parameters for $T = 303$ K are $\Delta H^\ddagger$ 15.5 kJ/mol, $\Delta G^\ddagger$ 335 kJ/mol, $\Delta S^\ddagger$ 1054 J·mol⁻¹·K⁻¹.

When MalP and Asn-133 → Ala are incubated in the presence of 300 mM P_i at, respectively, 45 and 60 °C for the time period of the assay used for the determination of the turnover numbers in Figure 2 (≈ 15 min), no irreversible loss of enzyme activity is detected for both proteins. Therefore, the values for the optimum temperature of catalysis by MalP and Asn-133 →

Figure 3 Deconvoluted FT-IR spectra of MalP at 20 °C

Spectra (A): the continuous line refers to the soluble part of MalP treated with 2 mM phosphate buffer, p²H 6.8. Dashed and dotted lines refer to MalP in 50 and 100 mM phosphate buffer, p²H 6.8, respectively. Spectra (B): continuous and dashed lines refer to the soluble and precipitated part of MalP in 2 mM phosphate buffer, p²H 6.8, respectively.

Figure 4 Temperature-induced aggregation of MalP

Light scattering was recorded at 30 °C and an emission wavelength of 550 nm with the excitation wavelength being 550 nm also. The normalized light-scattering intensities are shown for experiments in the absence of P_i (○) and the presence of 50 mM P_i (●) in 50 mM Tris buffer, pH 6.9. The holding time at each temperature was 5 min, and the heating rate between two temperatures of measurement was about 1 °C/min. The enzyme concentration was 0.4 mg/ml.

Ala do not reflect irreversible denaturation of these enzymes. They reflect one or more denaturation steps which occur at ≥ 40 °C for MalP and ≥ 55 °C for Asn-133 $\rightarrow$ Ala, and are fully and rapidly reversible on cooling both proteins to the temperature of the coupled phosphorylase assay (30 °C).

Structural characterization of MalP by FT-IR

When MalP was dissolved in $^2\text{H}_2\text{O}$ containing 2 mM phosphate, p ^2H 6.8, a large amount of protein precipitated and only a small fraction remained soluble. Precipitation appears to be triggered by the exchange of H_2O by $^2\text{H}_2\text{O}$ since even in the complete absence of P_i , the enzyme (1–10 mg/ml) was soluble in H_2O or in 50 mM Tris buffer, pH 6.9. The deconvolved spectra of the soluble part of MalP in 2 mM P_i , and of MalP in 50 or 100 mM P_i buffer are shown in Figure 3A. The amide I' band in $^2\text{H}_2\text{O}$ medium (1700–1600 cm^{-1} region) is very similar in all three samples, and thus the secondary structure composition of the enzymes in the samples. The intensity of the residual amide II band (1548.2 cm^{-1}) is lower in the spectrum of the soluble fraction of MalP in 2 mM P_i than in the spectra of the other two samples (Figure 3A). For proteins dissolved in $^2\text{H}_2\text{O}$, the residual amide II band intensity is a measure of the extent of $^1\text{H}/^2\text{H}$ exchange [25]. Therefore, this finding indicates that the solvent ($^2\text{H}_2\text{O}$) has a smaller accessibility to MalP in the presence of 50 mM and 100 mM P_i , compared with the soluble fraction of MalP in 2 mM P_i . The bands shown in Figure 3A can be assigned according to previous studies on proteins [26–31]. In particular, the 1653.8 cm^{-1} band is due to α -helical structure while β -sheets display a band at 1638 and 1639 cm^{-1} . The 1693.7 and the 1685.4 cm^{-1} bands can also be assigned to β -structure [32] while the bands at 1679.9 and 1671.7 cm^{-1} are probably due to turns [32]. The other bands displayed in Figure 3A may be assigned to amino acid side chain absorption [32,33].

Figure 3B shows that the secondary structure of precipitated MalP is quite different compared with that of the soluble part of the enzyme. In particular, the α -helix (1653.5 cm^{-1}) and β -sheet bands (1632.9 cm^{-1}) of precipitated MalP are lower in intensity and are present at lower wavenumber than in the spectrum of

soluble part of the enzyme. Moreover, the appearance of the 1642 cm^{-1} band indicates an increase of unordered structures in this insoluble sample. Figure 3B also shows that the residual amide II band is higher in the spectrum of precipitate than in the spectrum of the soluble part of MalP, indicating a lower $^1\text{H}/^2\text{H}$ exchange in the insoluble than in the soluble part of the enzyme.

Thermal denaturation of MalP monitored by absorbance spectroscopy and light scattering

Changes in the absorbance spectrum of MalP have been monitored during controlled thermal denaturation. Apart from the intrinsic protein absorbance at around 280 nm, the absorbance maximum of enzyme-bound PLP at 332 nm [22] and of free PLP at 405 nm can be used to monitor cofactor dissociation and denaturation. No significant changes in the absorbance spectrum of MalP occur between 25 and 40 °C. At 42 °C and in the absence of P_i , the absorbances at 281 and 450 nm increase dramatically, leading to the disappearance of the peak at 332 nm without a detectable increase in the absorbance at 405 nm. Between 42 to 44 °C, turbidity appears, and a colloid-like precipitate is formed. Aggregation of MalP starts in this temperature range, and that has been corroborated by light scattering measurements also (Figure 4). Phosphate has a small but significant effect on the temperature that triggers aggregation of MalP: in the presence of 50 mM P_i , aggregation starts between 44 and 47 °C (Figure 4, cf. Figure 2).

The failure to observe an increase in absorbance at 405 nm during denaturation of MalP suggests that dissociation of PLP does not precede the aggregation of the enzyme. However, to obtain firmer evidence on that point, MalP was denatured at 44 °C (50 mM Tris, pH 6.9). Aggregated protein was removed by centrifugation, and PLP in the supernatant was determined by using the method of Wada and Snell [23]. The PLP content in the precipitate was measured accordingly. The soluble and aggregated forms of MalP contain PLP in exactly the expected stoichiometry (1.1 ± 0.1 mol of PLP/mol of MalP subunit). PLP does not accumulate in the supernatant, even when about 80 % of the protein has already aggregated. Therefore, no release of cofactor occurs as result of thermal denaturation at 44 °C. (It cannot be excluded completely that MalP unspecifically incorporates the previously released PLP on aggregation, but that is a very unlikely possibility.) The soluble protein fraction has lost enzyme activity in a reversible manner. Therefore, (1) the reversibly inactivated MalP is the enzyme form that aggregates, and (2) aggregation is the step which is responsible for the irreversible loss of enzyme activity. When MalP is incubated in the presence of 50 mM P_i at 60 °C at which temperature melting of the secondary structure of the enzyme has been shown to take place by FT-IR (see below), PLP is released quantitatively into solution.

Thermal denaturation of MalP monitored by FT-IR

The increase in temperature leads to changes in the deconvolved infrared spectrum of MalP, typically indicative of thermal protein denaturation (Figure 5). A dramatic change of the amide I' bandshape occurs when the temperature is raised from 55 to 60 °C indicating in this temperature range, the major structural changes of MalP. At 60 °C, the α -helix and β -sheet bands, which are still well visible at 55 °C, disappear, and a broad band at about 1643 cm^{-1} , due to unordered structures, appears. Two strong peaks, close to 1617 and 1682 cm^{-1} , characterize also the spectrum at 60 °C. These two bands, which are smaller in the spectrum recorded at 55 °C, are due to intermolecular protein aggregation, triggered by protein denaturation and driven by

Figure 5 Thermal denaturation of MalP

Deconvolved FT-IR spectra of MalP in 50 mM phosphate buffer, p^2H 6.8, as a function of the temperature. Spectra are displayed at 5 °C intervals.

new hydrophobic interactions as a consequence of the denaturation [29,30,34]. The denaturation of MalP induces also a further $^1H/^2H$ exchange as shown by the decrease of the residual amide II band intensity at close to 1547 cm^{-1} .

More detailed information on temperature-dependent structural changes of MalP has been obtained from difference spectra by making the difference between two original absorbance spectra collected at close temperatures [35] (Figure 6). In particular, between 45 and 50 °C the intensity of a weak band at 1642.5 cm^{-1} increases, reflecting a slight increase of unordered structures, whereas the intensity of a band at 1541.5 cm^{-1} decreases, reflecting an increase in $^1H/^2H$ exchange. A tiny negative band seen close to 1656 cm^{-1} in the difference spectrum between 45 and 50 °C, indicates minor, presumably reversible modifications (losses) of the secondary structure of MalP in this temperature range. We recall the fact that when phosphate is present, reversible denaturation of MalP occurs at 40–45 °C, and the subsequent aggregation of the enzyme starts at ≈ 45 °C, as shown by measurements of enzyme activity, protein absorbance and light scattering (cf. Figure 4). That is partly corroborated by the difference spectra between 40 and 50 °C in Figure 6. However, in contrast to the results obtained by absorbance spectroscopy, no aggregation is observable by FT-IR at temperatures between 40 and 50 °C (see the Discussion section). The positive bands close to 1684 and 1618 cm^{-1} are due to protein aggregation which is seen to start between 50 and 55 °C, presumably reflecting the intermolecular protein aggregation also revealed in Figure 5. The bands at close to 1656 cm^{-1} and at 1541.5 cm^{-1} show maximum intensities between 55 and 60 °C, locating the overall melting of protein structure in this temperature range.

The increase in temperature leads also to other changes in the infrared spectrum, such as, for instance, amide I' band-shift and broadening [28]. The denaturation has been followed by monitoring as a function of the temperature, the amide I' bandwidth, calculated at $\frac{3}{4}$ of the amide I' band height ($W_{3/4H}$). Results are

Figure 6 Difference spectra between two original absorbance spectra of MalP recorded at different temperatures

Each difference spectrum is the result of the difference between two original absorbance spectra recorded in 50 mM phosphate buffer, p^2H 6.8, at the temperatures reported on the left of each difference spectrum (e.g. 40–45 °C). In the 1700–1600 cm^{-1} region, negative and positive bands represent the loss of secondary structure and protein aggregation, respectively. The negative band close to 1541 cm^{-1} is due to further $^1H/^2H$ exchange which occurred in the sample at higher temperature.

shown in Figure 7. MalP in 50 mM P_i has the same thermal stability as in 100 mM P_i , the temperature of denaturation (T_m) being observed for both samples at ~ 58 °C. The thermal denaturation curve of the insoluble part of 2H_2O -treated MalP shows a T_m at ~ 67 °C.

Figure 7 Melting of structure of MalP at elevated temperatures

Lines (a) and (b), which are almost indistinguishable, refer to MalP in 50 and 100 mM phosphate buffer, p^2H 6.8, respectively. Line (c) refers to the insoluble part of MalP in 2 mM phosphate buffer, p^2H 6.8.

DISCUSSION

Thermal denaturation of MalP

Scheme 1 describes the mechanism of thermal denaturation of MalP. The initial denaturation step is reversible and destroys the active site rather than the quaternary structure of the enzyme. This conclusion is strongly supported by two experimental findings. (1) The Asn-133 → Ala mutant is by approx. 15 °C more resistant to reversible thermal inactivation than wild-type MalP, and Asn-133 is in the active site and not at the interfacial area of MalP. (2) Upon incubation of MalP at 42–45 °C, recovery of reversibly lost enzyme activity must occur rapidly within the dead time of the assay for measuring phosphorylase activity. However, association of phosphorylase subunits to yield native enzyme has been studied during reconstitution of monomeric apo-phosphorylase in the presence of excess of PLP, such as to make cofactor binding not rate-limiting [36,37]. It is a slow process which *in vitro* requires at least several minutes for completion [36,37].

Apart from slight increases in unordered structure and $^1\text{H}/^2\text{H}$ exchange, reversible denaturation of MalP is accompanied by very small structural changes that can be monitored by FT-IR. Despite that fact, however, aggregation of the PLP-containing dimer is triggered by these conformational changes and starts in the temperature range 42–45 °C. Aggregation is the step responsible for the irreversible inactivation of the enzyme. Interestingly, exchange of H_2O by $^2\text{H}_2\text{O}$ seems to affect the enzyme in a similar way as does elevated temperature. Oxyanions such as phosphate or sulphate, and AMP stabilize against the aggregation which implies that they inhibit the irreversible step in the denaturation pathway of MalP. They have no significant effect on the reversible denaturation step. The failure of Glc 1-P to bring about any stabilization of MalP together with the complete absence of competition between Glc 1-P and phosphate concerning the stabilization of MalP provides strong evidence in support of the notion that the effect of phosphate results from MalP/oxyanion interactions that do not occur in the active site of the enzyme.

In contrast to the enzyme activity, the structural core of MalP is quite stable. Melting of the secondary structure is observed at ≈ 58 °C; that is, about 16 °C above the temperature at which reversible inactivation takes place. The overall loss of secondary structure is accompanied by the release into solution of the enzyme-bound cofactor, which is a process that is known to be

accompanied by the dissociation of the protein subunits. In addition, melting of the structural core of MalP occurs concurrently with new intermolecular protein/protein interactions which are typical of thermally or solvent denatured proteins [29,30,34], and are characterized by the appearance of two strong bands at close to 1620 and 1680 cm^{-1} in the FT-IR spectrum (cf. Figures 5 and 6). These interactions reflect a final aggregation step in the denaturation mechanisms of MalP, which involves formation of new β -sheet-like structures, manifested by these 1620 and 1680 cm^{-1} bands, as a probable consequence of the formation of new hydrophobic contacts caused by protein denaturation.

Driving force of the aggregation of MalP at elevated temperature and in $^2\text{H}_2\text{O}$

FT-IR analysis has shown that aggregation of MalP at ≈ 45 °C (at which temperature the secondary structure of MalP is stable) is not caused by new hydrophobic protein/protein interactions. Therefore, this implies that MalP aggregates as a consequence of interactions that are of an electrostatic, presumably ionic nature, and this kind of intermolecular interactions would not be visible in the infrared spectrum. The infrared spectrum of the precipitate of MalP, obtained in $^2\text{H}_2\text{O}$ (with 2 mM P_i), revealed a higher content of unordered structures, a lower intensity of the bands related to α and β structures and less $^1\text{H}/^2\text{H}$ exchange than in the spectrum of the soluble part. These data indicate that on aggregation, the secondary structure of the enzyme is destabilized and, as a consequence, part of the α and β structures are lost. However, during the modification of the secondary structure, a precipitate, with a high thermal stability, is formed whose structure melts at about 67 °C.

Stability and activity of wild-type MalP and the Asn-133 → Ala mutant

The Asn-133 → Ala mutant is 2-fold less active than the wild type. The effect is almost entirely on k_{cat} , and chemical inter-conversion of the ternary enzyme–substrate complex is assumed to be the rate-limiting step in glucan phosphorylase catalysis [38], reflected by the value of k_{cat} . The temperature-dependent increase of the turnover number of the mutant ($E_a \approx 18$ kJ/mol) is significantly smaller than that of MalP ($E_a \approx 84$ kJ/mol). The crystal structure of the Asn-133 → Ala mutant co-crystallized with oligosaccharide [4] suggests that any effect on k_{cat} induced by replacing Asn-133 with Ala will probably be indirect. Asn-133 does not seem to play a role in substrate binding or catalysis. However, it interacts via van der Waals contacts with Glu-88 which in turn is involved in interaction with the glucan substrate. Tyr-280 which makes stacking interactions with the oligosaccharide and van der Waals contacts with Glu-88, is also close to Asn-133. Interestingly, the 380s loop (residues 377–387) which is involved in an induced-fit conformational change on oligosaccharide binding to MalP is also affected by replacing Asn-133 with Ala [4,6]. Mutational [39] and structural [4–6] studies have pointed out an important contribution of residues in the 380s loop for efficient turnover (k_{cat}).

What is the reason for the fact that the mutant enzyme is more stable against thermal denaturation than the wild type? Deamidation of Asn residues is not a likely event to occur at 40–45 °C, and phosphate ions have been reported to inhibit Asn deamidation in lysozyme [40]. However, Asn-133 is part of a Gly-rich hexapeptide (Gly-Asn-Gly-Gly-Leu-Gly) of the sequence, and it has been shown that conformational flexibility, eventually brought about by glycines, can facilitate deamidation [41]. Deamidation with concomitant release of NH_3 does not

Scheme 1 Denaturation pathway of MalP

N is the native enzyme, D is the reversibly inactivated enzyme, and U is the unfolded protein that has lost secondary structure. The active-site bound PLP, the dimer structure of MalP, and the aggregation are indicated. Phosphate (P_i) is shown to inhibit the aggregation (see the Discussion section). D' indicates a slight modification of secondary structure on aggregation of the PLP-containing MalP dimer. The reversible transition $\text{N} \rightarrow \text{D}$ is triggered by elevated temperatures but also by exchange of H_2O by $^2\text{H}_2\text{O}$.

occur to a measurable extent at 45 °C, ruling out its contribution to inactivation of MalP. In a rather hydrophobic environment such as the active site of phosphorylase [3–6], the exchange of Asn by Ala could decrease nonfavourable interactions between the polar Asn and other nearby nonpolar amino acid residues, thereby increasing the stability of this active-site region. In addition, positional changes in the 380s loop could bring about extra stability. However, the stabilization is correlated with reduced catalytic efficiency, which could reflect the finely balanced active-site conformation of wild-type MalP with respect to flexibility, favouring fast turnover, and rigidity, favouring structural stability. Compared with wild-type MalP, the stability of the Asn-133 → Ala mutant is not due to a change in the mechanism of thermal denaturation. It reflects a drastic shift of the entire pathway to a ≈ 15 °C higher value on the temperature scale.

Structure of MalP and possible effect of phosphate on stability

Crystals of MalP unliganded with oligosaccharide have been obtained from phosphate-buffered solution (0.1 M P_i), and the structure of the enzyme at high resolution indicates occupancy of the active site by a phosphate molecule [3]. However, another phosphate dianion may be bound at a site that is created by two arginine residues, Arg-310 and Arg-316, whose guanidinium groups come close together in the MalP structure [3]. Arg-310 belongs to the $\alpha 8$ helix whereas Arg-316 is part of a short surface turn of four residues, leading to the $\alpha 8b$ helix. The $\alpha 8$ helix directly follows the 280s active-site loop (residues 280–287). Based on these considerations of MalP structure [3,4,6] and on recalling again the fact that stabilization of MalP by oxyanions is not brought simply by active-site interactions, a hypothetical model of thermal denaturation of MalP is as follows.

We propose that reversible denaturation of MalP reflects a conformational change of the 280s or 380s active-site loops, or of both. These small structural transitions would lead to a loss of a functional active site and presumably affect adjacent substructures such as the $\alpha 8$ helix, for example, and regions farther away in the MalP monomer. It has been shown that shifts in the 380s loop are correlated with shifts (> 1.5 Å) elsewhere in the molecule [4,6]. A consequence of the conformational change in the active site would be the exposure of aggregation-prone ionic protein surface, involving *inter alia* the $\alpha 8$ helix and the following short surface loop. Phosphate bound at the C-terminus of the $\alpha 8$ helix, by interactions with residues Arg-310 and Arg-316, could inhibit aggregation of MalP dimers, which in the absence of phosphate has been shown to occur rapidly. The effect of phosphate could be due to (i) compensation of the positive charge of the guanidinium groups at the proposed phosphate site, or (ii) introducing additional, site-specific repulsive interactions between MalP dimers, based on negative charge. Arg-310 and Arg-316 seem to create a relatively specific but low-affinity oxyanion site: (1) besides phosphate and sulphate, AMP is the only efficient stabilizer of MalP; interestingly, Arg-309 and Arg-310 make the 'phosphate gripper' of the AMP site in regulated glycogen phosphorylase [13–15]. (2) The effect of P_i on MalP stability at 45 °C shows a complex, not clearly saturable dependence on the phosphate concentration. In conclusion, regarding reversible and irreversible inactivation of MalP, the major 'weak' points of the protein include the active site and most probably, the adjacent $\alpha 8$ helix with the following short surface loop. The greater thermostability of the Asn-133 → Ala mutant, relative to wild-type MalP, can be assigned clearly to a sole stabilization of a functional active site. The model of thermal denaturation of MalP could provide a useful basis for further

studies on the stability of the enzymes by using site-directed mutagenesis since replacement of Arg-310 or Arg-316 can be predicted to abolish the stabilization by phosphate. In addition, it could be employed as structure-based frame of reference for comparing the stabilities of different α -glucan phosphorylases.

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