SHORT COMMUNICATIONS

DEHYDRATION OF MACROMOLECULES*

II.† PROTECTIVE EFFECTS OF CERTAIN ANIONS ON RIBULOSEDIPHOSPHATE CARBOXYLASE SUBJECT TO LOW WATER POTENTIALS IN VITRO

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Abstract

Studies of the instability of ribulosediphosphate carboxylase at low water potentials, generated in a pressure-membrane apparatus, have been continued. The presence of phosphate, pyrophosphate, citrate, or ribulose diphosphate during treatment protects the protein against fragmentation. Tris-sulphate, sodium sulphate, tetra-alkyl ammonium salts, or polyvinylpyrrolidone do not affford protection.

The results are considered in terms of water-structuring by ions, the binding of ions to the protein, and stabilizing of the protein by ions. Reported effects of ions on enzyme kinetics do not explain the difference between sulphate and phosphate with respect to protection. It is concluded that protective ions must be bound to cationic sites on the protein but must also structure adjacent water. The additional structured water presumably prevents fragmentation of the protein that can occur at low water potentials.

Introduction

A physical method employing a pressure-membrane apparatus was used by Darbyshire and Steer (1973) to manipulate the water potential of in vitro enzyme preparations. The activities of a number of enzymes after dehydration followed by rehydration, were reported. Ribonuclease and indoleacetic acid oxidase activities were unaffected by exposure to low water potentials. However, ribulosediphosphate carboxylase (E.C. 4.1.1.38) in Tris–sulphate or Tris–chloride buffers, lost activity when exposed to a range of water potentials from -2 to -14 bars. The loss of activity was accompanied by an apparent fragmentation of the protein as judged by Sephadex gel-filtration studies.

From the percentage recovery of the native protein, the influence of different ions present during dehydration of ribulosediphosphate carboxylase has been determined. It will be demonstrated that some anions afford considerable protection against dehydration in vitro and possible mechanisms are discussed. Finally the relevance to the in vivo situation has been assessed.

Methods and Materials

Ribulosediphosphate carboxylase was purchased from the Sigma Chemical Co. and used without further purification. After polyacrylamide-gel electrophoresis a major slow-moving band

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was detected with a minor slower band that was considered to be an aggregate (Trown 1965). On 0·1% SDS gels two bands consistent with the two subunit species were found. Ribulose diphosphate dibarium salt (Sigma Chemical Co.) was converted to the sodium salt with sodium sulphate. Tricine [N-tris(hydroxymethyl)methyl glycine], HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), Tris, and carbamyl phosphate were purchased from Calbiochem Pty. Ltd. and tetra-alkyl ammonium salts from Eastman Kodak Co.

Operation of the Pressure-membrane Apparatus.—The apparatus has been described by Darbyshire and Anlezark (1972) and its operation by Darbyshire and Steer (1973). In these experiments 0·3 ml buffer containing 0·5 mg ribulosediphosphate carboxylase was added to the chamber and a pressure of 7·3 bars was applied. After equilibration for 90 min, with the chambers maintained at 25°C, the pressure was released thus subjecting the protein phase inside the chamber to a water potential equivalent to the applied pressure, i.e. −7·3 bars, excluding the water potential contributed by the buffer solution. After 5 min the protein was rehydrated by the addition of 3 ml of buffer and 15 min later aliquots of the solution were removed for gel electrophoresis. Control gels were prepared from the original protein solution that had not been subjected to low water potentials.

Polyacrylamide-gel Electrophoresis.—Discontinuous electrophoresis in 7% polyacrylamide gels was carried out in the Tris–glycine system of Davis (1964) as reported by Darbyshire and Steer (1973). 100–170 µg protein were applied per gel and electrophoresis was at 3 mA per gel. Estimates of the native ribulosediphosphate carboxylase protein on the gels were made by the method of Racusen and Foote (1965). The bands stained with amido black were cut out and eluted with 3 ml 1 N NaOH at 37°C. The absorbance of the eluate was measured 600 nm. The amount of native protein recovered after low water potential treatment was expressed as a percentage of that recovered from control gels.

Ultraviolet Difference Spectra.—Matched tandem cells were used (Herskovits 1967) in a Perkin–Elmer 402 double-beam spectrophotometer.

<table>
<thead>
<tr>
<th>Ion</th>
<th>$\Delta S^0$</th>
<th>$B_{25^\circ C}$</th>
<th>Ion</th>
<th>$\Delta S^0$</th>
<th>$B_{25^\circ C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K+</td>
<td>16·5</td>
<td>(−0·007)</td>
<td>(CH$_3$)$_2$N$^+$</td>
<td>—</td>
<td>0·12</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>6·4</td>
<td>0·086</td>
<td>(C$_2$H$_4$)$_2$N$^+$</td>
<td>—</td>
<td>0·38</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>−36·2</td>
<td>0·039</td>
<td>(C$_3$H$_2$)$_2$N$^+$</td>
<td>—</td>
<td>0·86</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>5·2</td>
<td>(−0·007)</td>
<td>(C$_4$H$_4$)$_2$N$^+$</td>
<td>—</td>
<td>1·28</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>−3·6</td>
<td>0·209</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>−60</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Calculated from the equation $\Delta S_{\text{unitary}} = \Delta S^0 - 2R \ln M$, where $M$ is the number of moles of solvent per 1000 g solvent and $R$ is the gas constant. $\Delta S^0_{H^+}$ is taken as zero.

† Calculated from the equation $\eta/\eta_0 = 1 + Ac^{0.5} + Bc$, where $\eta$ is the viscosity of the solution, $\eta_0$ the viscosity of the solvent, and $c$ is the concentration of solute in moles per litre. $A$ and $B$ are constants. Arbitrarily designated values are in parentheses.

Results

It is convenient to consider the results with respect to (i) the water-structuring ability of the ions used; (ii) the ability of ions to bind with the protein; and (iii) the ability of the ions to stabilize the native protein conformation.

Table 1

**PARTIAL UNITARY MOLAR ENTROPY ($\Delta S^0$)* AND VISCOSITY CONSTANT ($B$)† VALUES FOR DIFFERENT IONIC SPECIES**

Values are taken from Von Hippel and Schleich (1969)
(i) Water-structuring Ability of Ions

Upon the addition of a small additional quantity of a solute to a solution a decrease in the partial molar entropy (the change in entropy resulting from the addition of solute to solution) reflects an increase in the structuring of the water surrounding the solute. In addition, viscosity $B$ constants that are positive reflect a structuring of water (Von Hippel and Schleich 1969). These parameters are listed in Table 1 for a number of different ionic species. It is evident that $PO_4^{3-}$ is highly effective in structuring water about it compared with the other ions listed in the table.

![Graph](image-url)

**Fig. 1.**—Percentage of native ribulosediphosphate carboxylase protein recovered after treatment at $-7.3$ bars as a function of the molarity of the buffer (pH 8·0). • Sodium or potassium phosphate. ■ Sodium pyrophosphate. ▲ Tris-sulphate. Standard errors of the means are shown.

The percentage recovery of native ribulosediphosphate carboxylase protein after dehydration and rehydration in sodium or potassium phosphate, sodium pyrophosphate, and Tris–sulphate at pH 8·0 and a range of concentrations is shown in Figure 1. Clearly, phosphate is most effective in protecting the protein from fragmentation: at 20 mM ($\equiv 11.8$ nmoles phosphate per milligram protein) 100% recovery of protein is achieved. The use of the sodium or potassium salt did not effect the results, suggesting that the anion was the effective agent. Sodium pyro-
phosphate was a less effective protectant and at 10 mM 64% native protein was recovered. When recovery was plotted against ionic strength (Fig. 2) both phosphate and pyrophosphate gave maximum protection at an ionic strength close to 0·05. Presumably the linear part of the phosphate and pyrophosphate plots reflect a progressive saturation of cationic sites on the protein. The less effective protection at ionic strengths greater than 0·05 is inexplicable at the moment. Percentage recovery of native protein in 50–300 mM Tris-sulphate (Fig. 1) was 10·0±1·4. Thus the relative protection afforded by phosphate and sulphate ions against dissociation of ribulosediphosphate carboxylase when the protein was dehydrated appeared related to the ability of these ions to structure water (see Table 1).

![Graph](image_url)

**Fig. 2.**—Percentage of native ribulosediphosphate carboxylase recovered after treatment at −7·3 bars (from Fig. 1) as a function of the ionic strength of the buffers. Ionic strength = Σez², where e is the concentration of an ion and z its charge. ● Sodium or potassium phosphate where the frequency of H₂PO₄⁻ and HPO₄²⁻ are taken as 3 and 97% respectively. ■ Sodium pyrophosphate where H₂P₂O₇³⁻: HP₂O₇²⁻: P₂O₇⁴⁻ = 5 : 70 : 25%. ▲ Tris-sulphate where 45% is ionized.

Other ions and compounds have been used to determine their ability to protect ribulosediphosphate carboxylase against the effects of low water potentials. In 50 mM sodium borate buffer, pH 7·8, recovery of protein was 34·7±12·6%, in 100 mM citrate buffer, pH 6·4, 91·7%. The presence of 6% (w/v) mannitol, 6% (w/v) sorbitol,
2 or 5% polyvinylpyrrolidone (PVP), or 75 mM carbamyl phosphate in Tris–sulphate gave protection values similar to Tris–sulphate alone. Also similar values were obtained from 50 mM tricine buffer, 180 mM HEPES buffer, 50 mM tetramethyl ammonium chloride in HEPES buffer, or 50 mM tetrabutyl ammonium bromide in HEPES buffer. Despite the water-structuring ability of the tetrabutyl ammonium ion (Table 1) and PVP (Klotz 1965), and the ability of mannitol and sorbitol to protect bacteria against lethal dehydration (Webb 1965) these agents have not protected the carboxylase at low water potentials.

(ii) Ion Binding

From kinetic studies phosphate is thought to bind to ribulose diphosphate carboxylase at the ribulose diphosphate active site (Trown 1965) and in our hands inhibits enzyme activity in a complex way with regard to ribulose diphosphate concentration (see Kawashima and Wildman 1970). Pyrophosphate is a more effective inhibitor and presumably binds in a similar way to phosphate. Thus in the presence of 0·5 mM ribulose diphosphate 50% inhibition was caused by 18·2 mM sodium phosphate or 4·6 mM sodium pyrophosphate. Carbamyl phosphate protects ribulose-diphosphate carboxylase from alkylation of the –SH groups (Argyroudi-Akoyunoglou and Akoyunoglou 1967) but its presence during low water potential treatment did not give protection against fragmentation. Sulphate also inhibits the enzyme (Trown 1965) in a manner similar to phosphate. However, sulphate when present as Tris–sulphate (Figs. 1 and 2) or as 300 mM Na₂SO₄ in HEPES buffer did not give protection. Thus effective protectants have the property of binding to ribulosediphosphate carboxylase but also have the property of being able to structure water (see Table 1).

From the results with phosphate it would be expected that ribulose diphosphate would also afford some protection to the enzyme exposed to low water potentials. This was the case but there was no effect of concentration over the range 1–30 mM in HEPES buffer. The mean percentage native protein recovered over that range was 84·9±1·8 (n = 9); only phosphate at 20 mM gave higher recovery.

The importance of the ionic environment to ribulosediphosphate carboxylase is emphasized by the behaviour of electrodialysed preparations. They were very difficult to solubilize and prolonged contact with buffer solutions resulted in very little protein going into solution. In addition, on exposure to relative humidities from 11 to 49%, an electrodialysed preparation took up eight times less water by weight than did untreated enzyme (B. Darbyshire, personal communication).

(iii) Protein Stability

The stability with time of pyruvate kinase is variably effected by monovalent cations (Wilson et al. 1967) and the ionic effects are paralleled by measurable perturbations of tryptophan residues in the protein. In ribulosediphosphate carboxylase, there was no indication from ultraviolet difference spectra of differential perturbation by Tris–sulphate, sodium phosphate, and ribulose diphosphate. However, measurement of the stability of enzyme activity in different buffers did show differences: 

\[ t_{1/2} = 60 \text{ min in Tris–chloride}; \quad 265 \text{ min in Tris–sulphate, and 2800 min in sodium phosphate}. \]

These values were obtained from one experiment only and are quoted to highlight the relative stabilities.
Discussion

Darbyshire and Steer (1973) suggested that ribulosediphosphate carboxylase was sensitive to low water potentials generated in the pressure-membrane apparatus because of the decisive role that water may play in the structure and stability of this protein, as compared to ribonuclease and indoleacetic acid oxidase. The results presented in this paper are consistent with the idea that to give protection, binding of solutes to a protein cationic site is necessary and that the bound species have water-structuring properties.

It may be argued that the only function of a charged species that binds to the protein is to remain on the protein side of the membrane in the pressure membrane apparatus. If binding does not occur ions and other solutes of small molecular weight will pass through the membrane during equilibration. However, PVP does not pass through the membrane and despite its water-structuring ability (Klotz 1965) it was ineffective in protecting ribulosediphosphate carboxylase from desiccation. Thus binding to a cationic site is a necessary prerequisite for protection.

In a study of the stabilization of the native conformation of ribonuclease by ions during heat denaturation it was concluded that ionic effects were not related simply to electrostatic or charge-shielding effects since ions showed different effects at the same ionic strength (Von Hippel and Wong 1964). This is also true for the ionic protection of the carboxylase against fragmentation, for at the same ionic strength the levels of protection by Tris–sulphate, pyrophosphate, and phosphate were quite different. However, both phosphate and pyrophosphate gave maximum protection at an ionic strength of 0·05 and at higher values were less effective.

A further case of stability conferred by ionic species is that of Nagradova and Guseva (1971) who studied the low-temperature dissociation of glyceraldehyde-3-phosphate dehydrogenase from rat skeletal muscle. They demonstrated that anions were variably effective in stabilizing the oligomeric enzyme and their order of effectiveness was related to their position in the Hofmeister lyotropic series. Fridovich (1963) has pointed out that the Hofmeister series is related to the differential interaction of the ions with water. Although a ranked list of effectiveness of protection is not possible in the present study, two conclusions have been reached: (1) the relative protective action of sulphate and phosphate, together with the protection by citrate, bear a superficial resemblance to the Hofmeister series; (2) the difference in their protective abilities may well reside in their relative water-structuring properties. Alternatively, the hydrated ionic dimensions or field intensity of the cationic site on the protein may account for the differences between sulphate, pyrophosphate, and phosphate.

The half-lives of enzyme activity in different buffer systems agree with the findings of Trown (1965) who suggested that ions that afforded stability to ribulosediphosphate carboxylase with time were those that had similar properties to the substrate, ribulose diphosphate. This suggestion is attractive in the present case since ribulose diphosphate does give protection to ribulosediphosphate carboxylase against the effects of low water potentials. The results reported here modify the conclusions of Darbyshire and Steer (1973) that ribulose diphosphate does not afford protection to ribulosediphosphate carboxylase activity at low water potentials. However, it does not explain why the sulphate ion or carbamyl phosphate are not effective
protectants. Sulphate has been found to inhibit ribulosediphosphate carboxylase activity in a manner similar to phosphate (Trown 1965; Paulsen and Lane 1966) although it is not clear whether either is competitive or non-competitive with respect to ribulose diphosphate (Kawashima and Wildman 1970). It was reported (Argyroudi-Akoyunoglou and Akoyunoglou 1967) that 3 mm carbamyl phosphate did not inhibit ribulosediphosphate carboxylase activity but we have found that in the presence of 0·5 mm ribulose diphosphate 50% inhibition was caused by 6·6 mm carbamyl phosphate. For comparison, the value for sodium phosphate was 18·2 mm; for sodium pyrophosphate 4·6 mm; for sodium sulphate 21 mm; and sodium citrate 9 mm. Thus enzyme inhibition and apparent binding to an active site (as distinct from general cationic sites) is not sufficient to distinguish protectants from non-protectants nor to explain the mechanism of protection.

Darbyshire and Steer (1973) concluded that while ribulosediphosphate carboxylase is very sensitive in vitro, the chloroplastic environment offers protection in vivo and this is supported by the results reported here. Santarius and Heber (1965) calculated the inorganic phosphate content of chloroplasts for a number of species. They reported a range from 4 to 25 mm and it is within this range that phosphate afforded maximum protection to spinach ribulosediphosphate carboxylase in vitro (Fig. 1). Moreover, the inorganic phosphate content might be expected to increase during periods of water stress since photophosphorylation is sensitive to water stress in vivo (Nir and Poljakoff-Mayber 1967; J. S. Boyer, personal communication). In addition, other proteins and ions in the chloroplast could add to the structuring of water and probably little of the water would be in a “free” condition (see, for example, Cope 1972).

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References


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