Stability Studies on Maize Leaf Phosphoenolpyruvate Carboxylase: The Effect of Salts†

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ABSTRACT: The solution stability of phosphoenolpyruvate carboxylase (PEPC) has been determined in the presence of various salts by temperature-accelerated enzyme inactivation and also by using high-performance size-exclusion chromatography. Kosmotropic (water structuring) anions in the Hofmeister series (HPO_4^{2-}, citrate^{3-}, SO_4^{2-}, F^-, OAc^-) and glutamate stabilized the enzyme most effectively, while Cl^- (a borderline Hofmeister anion) and Br^- (a chaotropic anion) were destabilizing. The effects of the cations on PEPC stability ranged from relatively inert (Na^+, K^+) to destabilizing ($((CH_3)_4N^+$, NH_4^+, Li^+). The observed stabilization of PEPC by specific salts has been interpreted in terms of the positive surface tension increment and the water-structuring effects conferred on the solution by the specific stabilizing reagents. Both these effects enhance hydrophobic interactions of proteins and increase the energy required to enlarge the surface area of the solvent cavity in which the protein resides. The destabilization of PEPC by some salts at a concentration of 0.5 M was associated with the dissociation of the tetrameric enzyme into its dimeric and monomeric forms, a process most probably occurring as a result of ion−peptide dipole binding, which promotes protein−solvent interaction and a subsequent reduction in the free energy of cavity formation. The stabilization of enzyme activity by kosmotropic salts depended on the salt concentration with maximum stabilization of PEPC in solution at 52 °C observed with 0.6−0.8 M sodium glutamate, 2 M KF, and 2.2 M KOAc. Higher concentrations of these salts resulted in decreased activity. This reduction in activity of PEPC in the presence of high concentrations of kosmotropic salts appears to be associated with irreversible conformational changes of the tetrameric enzyme.

Phosphoenolpyruvate carboxylase (PEPC)[1] from *Zea mays* is a homotetrameric enzyme with a molecular mass of approximately 400 kDa (Uedan & Sugiyama, 1976; Wagner et al., 1987). The relatively low stability of PEPC in solution has been attributed to the tendency of the tetramer to dissociate readily into dimers (approximately 200 kDa) with reduced activity and into monomers (approximately 100 kDa) with almost no activity (Wagner et al., 1987; McNaughton et al., 1989). The enzyme contains one active site per subunit (Wagner et al., 1987). Subunit interaction affecting the active sites is considered to be responsible for the progressive increase in specific activity in the dimeric and tetrameric forms of PEPC (Wagner et al., 1988; Jawali, 1990a,b). The dissociation in solution of the tetrameric enzyme to the monomer is believed to be a two-step process, characterized by a reversible dissociation of the tetramer to the dimer, with a subsequent, essentially irreversible dissociation from the dimer to the monomer (Wu et al., 1990). Dynamic light scattering measurements by Wu and co-workers (1990) have also shown that, following the dissociation of PEPC into the monomeric form, the monomers associate into large and inactive aggregates, which may well account for the marked instability of the isolated monomeric form of the enzyme.

Because PEPC is widely used in clinical chemistry, methods to stabilize solution preparations of the enzyme when held at elevated temperatures or for long periods of time are highly desirable. Currently little is known about the conditions of buffer composition which are necessary to achieve this outcome. In this paper the effects of high concentrations of several salts on the stability of PEPC in solution are described. The results are discussed within the context of the activity profile of this enzyme and possible mechanisms of stabilization.

MATERIALS AND METHODS

Phosphoenolpyruvate carboxylase was purchased from Sorrento Biochemical Inc. (San Diego, CA). The enzyme preparation contained bovine serum albumin (BSA) as stabilizer. All reagents purchased were of the purest grade available and were obtained from either Merck Chemicals (Melbourne, Australia) or Sigma-Aldrich (St. Louis, MO).

PEPC Activity Assay. PEPC activity was measured at 30 °C with a Cobas bio-centrifugal analyzer, by following the conversion of NADH to NAD^+ at 340 nm, in an assay medium containing Tris-HCl (53 mM), $MgCl_2$ (6.5 mM), $NaHCO_3$ (10 mM), *N*-Acetylcysteine (286 mM), NADH (0.23 mM), PEP (5.7 mM), and malate dehydrogenase (630 munits/mL), adjusted to pH 8.50 with 5 M NaOH. All PEPC samples, except for fractions collected by HPSEC, were diluted 10-fold with 20 mM MES/5 mg/mL BSA (pH 6.40)

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[1] Abbreviations: PEPC, phosphoenolpyruvate carboxylase; BSA, bovine serum albumin; NADH, nicotinamide adenine dinucleotide; MES, 2-(*N*-morpholino)ethanesulfonic acid; HPSEC, high-performance size-exclusion chromatography; HPLC, high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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immediately prior to assay. The overall dilution of the PEPC test samples following incubation with the different salts in the thermal stability studies, when placed in the final assay medium, was 500-fold. Fractions of the PEPC-containing eluent collected from the HPSEC experiments were diluted 50-fold in the final assay medium prior to the PEPC activity assays. Activity measurements are reported as the mean ± SEM of replicate samples.

PEPC Stability Profiles in the Presence of Test Reagents. All pH measurements were performed at 20 ± 2 °C. Reagent stock solutions were diluted to a specified concentration with 1 M MES and water to obtain a final concentration of either 20 or 100 mM MES and a PEPC content of 20 mg/mL. The pH adjustments of the reagent solution were performed with the addition of acid (usually with the acid corresponding to the anion of the salt under investigation, otherwise 2 M HCl) or base (5 M NaOH), such that the 2-fold dilution of the reagent solution with 40 mg/mL PEPC and 20 or 100 mM MES (pH 6.30) produced a final test sample at the optimal pH (6.30) for incubation at 50 °C. The change in MES buffer concentration (20 or 100 mM MES) had no significant effect on the stability of PEPC under test conditions. For the fluoride salts, the protonated form of Dowex 50W cation-exchange resin was used for acidic pH adjustment. The pH of the various test samples so prepared fell within ±0.1 pH unit of the pH value for maximal enzyme stability at the temperature of incubation. For experiments involving the determination of the optimal concentration profiles of the stabilizing reagents with incubation at 52 °C, samples were prepared by the 5-fold dilution of a 100 mg/mL PEPC stock solution in 100 mM MES (pH 6.20) with the reagent solution to a final concentration of 20 mg/mL PEPC in the test sample (~pH 6.20). The pH at which the test samples were prepared corresponded to the pH at which PEPC was maximally stabilized and active at the temperature of the incubation (i.e., at pH 6.30 at 50 °C and pH 6.20 at 52 °C). After incubation at 50 or 52 °C, the test samples were chilled in iced water (ca. 5 °C) and then assayed for activity. Test samples were centrifuged to remove any precipitate, and the supernatants were assayed for activity to assess the salting out capacity of a particular reagent toward PEPC.

High-Performance Size-Exclusion Chromatography (HPSEC) Experiments. HPSEC experiments were performed on a Beckman System Gold HPLC system, comprising a Beckman 166 variable wavelength detector and a Beckman 126 dual pump solvent module with a DuPont GF-250 column (250 × 9.4 mm i.d.). The eluent was monitored at 280 nm. Samples were filtered through Sartorius 0.5-μm cellulose nitrate filters prior to injection. The column was calibrated with protein molecular mass standards for each mobile phase used, since protein retention times were noted to vary quite significantly between different eluents. Protein standards included ovalbumin (46 kDa), BSA (67 kDa), BSA dimer (134 kDa), yeast alcohol dehydrogenase (150 kDa), catalase (232 kDa), ferritin (440 kDa), β-galactosidase (540 kDa), and thyroglobulin (669 kDa). Values of molecular mass and distribution coefficient were derived according to the method of Himmel and Squire (1981). The flow rate during the experimental runs was 0.5 mL/min; chromatography was performed at room temperature (~20 °C), with an experimental run time of 30 min. PEPC samples (20 mg/mL) were dissolved in the chromatographic mobile phase being studied and then filtered prior to sample injection. The volume of the PEPC sample injected was 40 μL during all experimental runs; 125 μL fractions were collected manually and kept on ice prior to the assay of enzyme activity.

SDS–PAGE. Polyacrylamide gel electrophoresis was performed on Pharmacia Phast equipment, using the procedure described in PhastSystem Separation Technique File No. 110. Staining was performed according to PhastSystem Development Technique File No. 210 (Silverstain). Molecular mass standards used for Phast gels included carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (67 kDa), and phosphorylase a (94 kDa). SDS–PAGE analysis was performed on the relevant fractions obtained from the HPSEC experiments and the temperature incubation experiments.

RESULTS

Effect of Temperature and pH on the Activity of PEPC. At temperatures between 4 and 25 °C, the relative activity of PEPC following incubation in a 20 or 100 mM MES solution in the presence or absence of a range of salts at different concentrations was found in preliminary experiments to undergo only small changes over a period of several days. Accordingly, the effects of various salts on the stability of the enzyme were studied using accelerated thermal inactivation. This experimental approach enabled the activity of different preparations of PEPC to be measured under standard assay conditions with a Cobas bio-centrifugal analyzer following incubation of the enzyme with the various salts at elevated temperatures chosen to ensure adequate thermal inactivation. Moreover, the experimental conditions were chosen so that significant dilution of the PEPC samples occurred following exposure of the enzyme to the various salts at elevated temperatures, thus eliminating the effect of the salts on the measurement of PEPC activity during the assay and/or following the short period of time while these diluted samples were stored in iced water prior to analysis. On the basis of these results, temperatures in the range 50–52 °C were chosen to produce adequate inactivation of different preparations of PEPC in MES buffer solutions in the absence of salts, i.e., ca. 35–50% over a period of 60 min. The effect of pH on the thermal inactivation of PEPC in solution was also examined over the range pH 5.8–6.7 at 50 and 52 °C as part of these initial investigations. Maximum retention of PEPC activity at 50 °C occurred at pH 6.30, while the corresponding value at 52 °C was pH 6.20. These temperature and pH values were used for all subsequent studies on the effects of salts on PEPC stability.

Effect of Reagents on the Stability of PEPC. In Table 1 the relative stabilities of PEPC in a 20 or 100 mM MES buffer at 50 °C in the presence of fluoride, chloride, and bromide salts at different concentrations in the range 0–2 M are shown. These experimental conditions permitted examination of the stabilization of PEPC by these halide anions in terms of their relative position in the Hofmeister or water structuring sequence of anions. It was clear from the experimental data that, over the range of salt concentrations studied, the fluoride salts had the greatest stabilizing effect at high concentrations (particularly at 2 M) relative to the incubation controls, whereas chloride and bromide salts were destabilizing. It was also apparent that all of the chloride salts and some of the fluoride salts (most notably NH_4F) had a destabilizing effect at or below 0.5 M.

Table 1: Effect of Univalent Halide Salts on the Stability of PEPC Incubated for 60 min at 50 °C[a]

cation	anion rel act.[b] F^-	Cl^-	Br^-	concn (M)
Li^+		0	0	2
		0	0	0.5
		0.60 ± 0.03	0.69 ± 0.01	0.05
Na^+		0.04 ± 0.00	0	2
		0.04 ± 0.00	0	0.5
	1.23 ± 0.02			0.45[c]
	1.06 ± 0.02	0.82 ± 0.03	0.83 ± 0.02	0.05
K^+	2.15 ± 0.03		0	2
		0.14 ± 0.01		1.6[c]
	1.04 ± 0.01	0.05 ± 0.00	0	0.5
	1.08 ± 0.02	0.77 ± 0.03	0.82 ± 0.01	0.05
Rb^+		0.39 ± 0.02		2
		0.05 ± 0.01		0.5
		0.78 ± 0.04		0.05
Cs^+		0.03 ± 0.00		2
		0.02 ± 0.01		0.5
		0.80 ± 0.04		0.05
NH_4^+	1.83 ± 0.02	0	0	2
	0.31 ± 0.01	0	0	0.5
	1 ± 0.01	0.75 ± 0.07	0.72 ± 0.01	0.05
$(CH_3)_4N^+$		1.09 ± 0.04	0	2
	2.18 ± 0.03			1.8[c]
			0	1.07[c]
	0.91 ± 0.01	0.05 ± 0.00	~0	0.5
	1.09 ± 0.01	0.78 ± 0.03	0.76 ± 0.01	0.05

[a] For experimental details see text. [b] Results are expressed as activity relative to that of an enzyme sample incubated under the same conditions of elevated temperature, without added salt, for 60 min. The activity of the control incubation samples at 50 °C and 60 min was 42.5 ± 1.2%, while the value at 50 °C and 0 min with salt added was 100% of the value of the control samples kept at 5 °C for 60 min. The mean original activity for PEPC samples kept at 5 °C in the above experiments was 1.94 ± 0.12 units/mg of powder. [c] Maximal salt concentration obtainable by method of test sample preparation.

Table 2: Effect of Sulfate and Phosphate Salts on the Stability of PEPC Incubated for 60 min at 50 °C

test salt	concn (M)	mean act. (units/mg)	% original act.[a]	rel act.[b]
sulfate temp controls (5 °C)		1.99 ± 0.01	100	2.09 ± 0.04
sulfate incubation controls (50 °C)		0.95 ± 0.02	47.9 ± 1.0	1
Li_2SO_4	0.68	0.49 ± 0.01	24.5 ± 0.6	0.51 ± 0.02
	0.48	0.05 ± 0.00	2.4 ± 0.2	0.05 ± 0.00
	0.05	0.69 ± 0.03	34.7 ± 1.3	0.73 ± 0.03
Na_2SO_4	0.94	1.66 ± 0.02	83.3 ± 1.1	1.74 ± 0.04
	0.5	1.46 ± 0.02	73.4 ± 1.0	1.53 ± 0.04
	0.05	0.90 ± 0.02	45.2 ± 0.8	0.94 ± 0.03
K_2SO_4	0.24	0.98 ± 0.01	49.2 ± 0.4	1.03 ± 0.02
	0.05	0.99 ± 0.01	49.8 ± 0.5	1.04 ± 0.02
$(NH_4)_2SO_4$	1.24	1.42 ± 0.00	71.4 ± 0.4	1.49 ± 0.03
	0.48	0.41 ± 0.03	20.5 ± 1.3	0.43 ± 0.03
	0.05	0.79 ± 0.03	39.4 ± 1.6	0.82 ± 0.02
$((CH_3)_4N)_2SO_4$	0.5	0.15 ± 0.00	7.3 ± 0.1	0.15 ± 0.00
	0.05	0.87 ± 0.02	43.6 ± 1.2	0.91 ± 0.03
temperature controls (5 °C)		1.72 ± 0.01	100	2.80 ± 0.04
phosphate incubation controls (50 °C)		0.61 ± 0.01	35.7 ± 0.4	1
K_2HPO_4	1.75	1.55 ± 0.00	90.5 ± 0.5	2.54 ± 0.03
	0.5	0.98 ± 0.01	57 ± 0.4	1.60 ± 0.02
	0.05	0.38 ± 0.01	22.2 ± 0.5	0.62 ± 0.01
$(NH_4)_2HPO_4$	1.6	1.39 ± 0.06	81.1 ± 3.5	2.27 ± 0.10
	0.5	0.34 ± 0.00	19.5 ± 0.2	0.55 ± 0.01
	0.05	0.33 ± 0.00	19.0 ± 0.2	0.53 ± 0.01

[a] % original activity is calculated as the percentage of enzyme activity relative to that of enzyme control samples kept at 50 °C for 0 min and 5 °C for 60 min with added salt. Differences in original activity and incubation control % original activity between incubation runs (compare Tables 1−5) were a consequence of variations between enzyme batches utilized in this study. [b] Relative activity is calculated as enzyme activity relative to that of enzyme samples incubated at 50 °C for 60 min without added salt.

Table 3: Effect of the Citrate Salts on the Stability of PEPC, Incubated for 60 min at 50 °C[a]

test salt	concn	mean act. (units/mg)	% original act.	rel act.
temperature controls (5 °C)		2.11 ± 0.02	100	2.83 ± 0.11
incubation controls (50 °C)		0.75 ± 0.03	35.4 ± 1.4	1
$Li_3Citrate$	1.16	1.36 ± 0.06	64.2 ± 2.8	1.82 ± 0.10
	0.5	0.06 ± 0.00	2.6 ± 0.1	0.07 ± 0.01
	0.05	0.28 ± 0.00	13.2 ± 0.2	0.37 ± 0.02
$Na_3Citrate$	0.77	1.80 ± 0.05	85.1 ± 2.5	2.41 ± 0.11
	0.5	1.72 ± 0.02	81.2 ± 1.3	2.29 ± 0.09
	0.05	0.40 ± 0.01	19.0 ± 0.5	0.54 ± 0.03
$K_3Citrate$	1.23	1.59 ± 0.04	75.1 ± 2.1	2.12 ± 0.10
	0.5	1.74 ± 0.01	82.1 ± 1.0	2.32 ± 0.09
	0.05	0.47 ± 0.00	22.2 ± 0.3	0.63 ± 0.03
$(NH_4)_2Citrate$	0.94	1.53 ± 0.04	72.6 ± 2.2	2.05 ± 0.10
	0.5	0.89 ± 0.01	42.2 ± 0.7	1.19 ± 0.05
	0.05	0.30 ± 0.00	14.4 ± 0.2	0.41 ± 0.02

[a] For further details, see Table 2.

With the exception of lithium and tetramethylammonium sulfates, the sulfate and phosphate salts tested had a stabilizing effect on PEPC at moderate to high concentrations (Table 2). Potassium and sodium sulfates had little or no effect on PEPC stability at a concentration of 0.05 M, although the corresponding phosphate salts were destabilizing at this concentration. Ammonium sulfate and ammonium phosphate were destabilizing at concentrations up to 0.5 M, but they stabilized PEPC at the highest concentrations tested. Lithium and tetramethylammonium sulfates destabilized PEPC at all concentrations tested. It is evident that the cations and anions exert their effects on the stability of PEPC independently. Sulfate and phosphate ions were generally effective stabilizers of PEPC activity, yet when these anions were paired with destabilizing cations such as lithium and tetramethylammonium, the stability of PEPC was substantially reduced.

Citrate salts stabilized PEPC at high concentrations, but were destabilizing at a concentration of 0.05 M (Table 3). As noted for the sulfate salts, destabilizing effects of lithium and ammonium citrate were observed at concentrations up to 0.5 M. The effects of acetate salts on PEPC stability are shown in Table 4 and Figure 1. Sodium and potassium acetates stabilized the enzyme at all concentrations above 0.05 M and at higher concentrations provided almost complete protection against thermal inactivation at 50 °C. Lithium and ammonium acetates destabilized the enzyme at concentrations up to 0.5 M; at higher concentrations (>1 M) these salts stabilized PEPC. In contrast to the destabilizing effects of most tetramethylammonium salts tested, tetramethylammonium acetate stabilized the enzyme activity at the concentrations tested. Table 5 shows that incubation with the monosodium salt of glutamic acid resulted in marked stabilization of PEPC over the concentration range tested (0.05−1.1 M).

Effect of Reagent Concentration. Additional studies on the effect of reagent concentration on PEPC stability were

Table 4: Effect of Acetate Salts on the Stability of PEPC Incubated for 60 min at 50 °C[a]

test salt	concn (M)	mean act. (units/mg)	% original act.	rel act.
temp controls (5 °C)		2.28 ± 0.01	100	2.47 ± 0.02
incubation controls (50 °C)		0.92 ± 0.00	40.5 ± 0.2	1
LiOAc	2	1.85 ± 0.02	81.3 ± 0.8	2.01 ± 0.02
	0.5	0.10 ± 0.01	4.5 ± 0.2	0.11 ± 0.01
	0.05	0.75 ± 0.00	32.9 ± 0.2	0.81 ± 0.00
NaOAc	2.5	2.20 ± 0.02	96.8 ± 1.1	2.39 ± 0.03
	2.0	2.18 ± 0.03	95.9 ± 1.2	2.37 ± 0.03
	1.5	2.14 ± 0.00	93.9 ± 0.5	2.32 ± 0.01
	1.0	1.90 ± 0.02	83.2 ± 1.0	2.05 ± 0.05
	0.5	1.28 ± 0.01	56.1 ± 0.4	1.39 ± 0.01
	0.05	0.92 ± 0.00	40.3 ± 0.2	1.00 ± 0.00
KOAc	3.0	1.93 ± 0.03	84.6 ± 1.4	2.09 ± 0.04
	2.5	2.12 ± 0.01	92.9 ± 0.7	2.29 ± 0.02
	2.0	2.06 ± 0.00	90.5 ± 0.4	2.23 ± 0.01
	1.5	2.06 ± 0.03	90.6 ± 1.3	2.24 ± 0.03
	1.0	1.74 ± 0.01	76.5 ± 0.5	1.89 ± 0.01
	0.5	1.24 ± 0.01	54.5 ± 0.4	1.35 ± 0.01
	0.05	0.92 ± 0.01	40.6 ± 0.3	1.00 ± 0.01
NH_4OAc	2	1.66 ± 0.03	72.9 ± 1.4	1.80 ± 0.03
	0.5	0.18 ± 0.00	8.0 ± 0.2	0.20 ± 0.00
	0.05	0.81 ± 0.01	35.4 ± 0.4	0.87 ± 0.01
$(CH_3)_4NOAc$	2	1.04 ± 0.03	45.8 ± 1.1	1.13 ± 0.03
	0.5	1.31 ± 0.02	57.4 ± 0.9	1.42 ± 0.02
	0.05	0.94 ± 0.00	41.1 ± 0.2	1.01 ± 0.01

[a] For further details, see Table 2.

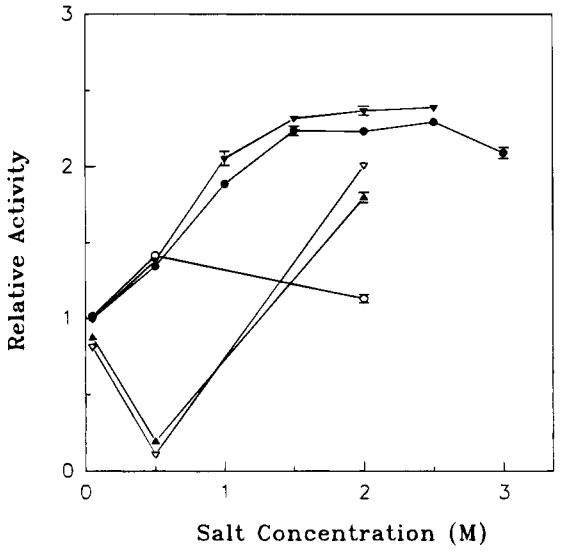

FIGURE 1: Reagent stability profiles for PEPC, incubated with acetate salts at 50 °C for 60 min: (▽) LiOAc, (▼) NaOAc, (●) KOAc, (▲) NH_4OAc, and (○) $(CH_3)_4NOAc$. Activity is expressed relative to that of the incubation control samples at 50 °C for 60 min without added salt, such that the incubation controls at 50 °C and 0 min have a relative value of 1.

carried out with various salts which had been found to substantially increase the stability of the enzyme (Figure 2). The temperature of incubation was elevated to 52 °C in these studies, to ensure significant losses in PEPC activity at all salt concentrations. The effects of salts which caused salting out of PEPC are presented in Table 6. Salts of the monovalent anions, including KF, $(CH_3)_4NF$, NaOAc, and KOAc (Figure 2A) had their maximum stabilizing effect on PEPC in the concentration range 2−2.5 M. Sodium glutamate was an exception, with a maximum stabilizing action over the range 0.6−1 M. Salts of polyvalent anions, including

Table 5: Effect of Monosodium Glutamate on the Stability of PEPC Incubated for 60 min at 50 °C[a]

test salt	concn (M)	mean activity (units/mg)	% original act.	rel act.
temp controls (5 °C)		2.36 ± 0.02	100	2.25 ± 0.03
incubation controls (50 °C)		1.05 ± 0.01	44.4 ± 0.6	1
NaGlutamate	1.09	2.26 ± 0.05	95.6 ± 2.4	2.15 ± 0.06
	0.5	2.32 ± 0.02	98.2 ± 1.2	2.21 ± 0.03
	0.05	1.84 ± 0.01	78.0 ± 0.9	1.76 ± 0.02

[a] For further details, see Table 2.

potassium citrate, potassium phosphate, and sodium sulfate, all achieved maximal stabilization at concentrations below 2 M. At their maximally stabilizing concentrations, the salts presented in Figure 2 had a very similar effect on the maintenance of PEPC activity, with a 6−12-fold retention of PEPC activity over the control level. Potassium phosphate had the greatest stabilization effectiveness, with 90.5 ± 0.6% of PEPC original activity remaining after 1 h at 52 °C in the presence of 1.75 M salt.

High-Performance Size-Exclusion Chromatography. Molecular weight calibration plots were generated for the DuPont GF-250 size-exclusion chromatographic experiments with the mobile phases containing each of the various salts at different concentrations. Measurement of these discrete calibration plots were necessary because the relative elution positions of the calibrating proteins varied from one salt system to another. A representative calibration profile, with 0.1 M MES (pH 6.70) containing 0.5 M NaCl as the eluent, is shown in Figure 3A. The elution times of several calibration proteins deviated from those expected on the basis of linearity in their distribution coefficient (K_D) versus log(MW) dependencies. BSA and BSA dimer consistently eluted earlier than predicted on the basis of their known molecular masses, under all mobile phase conditions. In addition, the relative elution positions of the tetrameric, dimeric, and monomeric forms of PEPC were also very sensitive to the presence of different salts; for example, the PEPC tetramer eluted as a protein with an apparent molecular mass of 363 kDa in the presence of 0.5 M NaOAc, but as a protein with an apparent molecular mass of 437 kDa in the presence of 0.5 M KOAc. Table 7 provides data on the size-exclusion chromatography behavior of PEPC in terms of the calculated apparent molecular mass of PEPC and its subunits, derived from the UV absorbance and enzyme activity measurements of the elution profiles under the various mobile phase conditions. Due to the presence of BSA and BSA multimers in the PEPC preparations, the various UV absorbance maxima of these elution profiles were slightly displaced to longer retention times, as a consequence of the peak overlap of the PEPC subunit and BSA absorbance profiles, from the elution positions of the PEPC species as determined from the measurement of enzymatic activity. The apparent molecular mass estimates for the PEPC dimer calculated from these UV absorbance profile maxima consequently diverged slightly from the values calculated from the activity profile measurements.

The elution profile for a PEPC preparation containing BSA in the presence of 0.5 M NaCl (Figure 3B) indicated the presence of an active PEPC component with an apparent molecular mass of ~214 kDa (PEPC dimer) and a second,

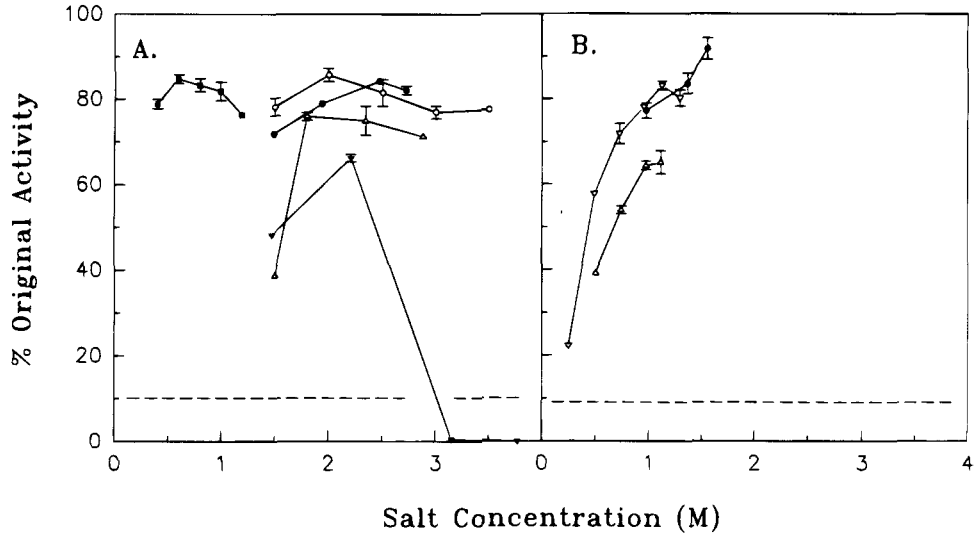

FIGURE 2: Salt concentration profiles for the maximal stabilization of PEPC with the salts of monovalent anions and glutamate (A) and polyvalent anions (B). Samples were incubated for 60 min at 52 °C. Panel A: (○) KF, (△) $(CH_3)_4NF$, (●) NaOAc, (▼) KOAc, and (■) NaGlutamate. Panel B: (▽) K_3Citrate, (●) K_2HPO_4, and (△) Na_2SO_4. Activity is expressed relative to that of the temperature controls, kept at 5 °C during the incubation period. (- - -) Incubation control activity.

Table 6: Homogenized and Supernatant Sample Activity for PEPC with Salts Producing Turbid Solutions at 5 °C, after Incubation for 60 min at 52 °C[a]

test salt	concn (M)	% original act. in homogenized sample	% original act. in pooled sample supernatant
KF	3.5	77.6 ± 0.7	24.7
	3	77.0 ± 1.5	9.6
	2.5	81.5 ± 3.1	39.8
	2	85.7 ± 1.5	80.7
	1.5	78.2 ± 2.1	82.0
KOAc	3.78	0.2 ± 0.1	0.9
	3.15	0.5 ± 0.3	1.6
	2.21	66.2 ± 0.9	79.2
	1.48	48.2 ± 0.8	62.3
Na_2SO_4	1.11	65.2 ± 2.7	49.0
	0.97	64.4 ± 1.0	55.7
	0.74	54.0 ± 0.9	56.3
K_2HPO_4	1.56	91.8 ± 2.5	22.2
	1.36	83.7 ± 2.4	30.2
	0.97	77.3 ± 1.8	72.8
$(CH_3)_4NF$	2.88	71.3 ± 0.5	65.7
	2.34	75.0 ± 1.2	71.9

[a] % original activity is calculated as the percentage of enzyme activity relative to that of enzyme control samples kept at 52 °C for 0 min and 5 °C for 60 min with added salt. See Table 2 for further details on the experimental conditions.

less active component of ~89 kDa (PEPC monomer). However, the total activity of the PEPC sample was significantly reduced compared to that in the control experiment lacking 0.5 M NaCl. Moreover, the enzyme activity and optical absorbance profiles derived from these chromatographic experiments indicated that the higher molecular mass species which eluted in the region corresponding to the tetramic form of PEPC and its aggregates was inactive. These results were confirmed by SDS−PAGE electrophoresis of the various chromatographic fractions obtained (Figure 4). PEPC was found in the chromatographic fractions corresponding to the elution positions of tetramer, dimer, and monomer (lanes 1−3, respectively). From the density of the band staining of the SDS−PAGE gels, the most abundant form of the enzyme under 0.5 M NaCl incubation/elution conditions was the dimeric form of PEPC. The absence of significant levels of activity in the eluted fractions which corresponded to the tetrameric form of PEPC may be a consequence of the destabilization of the enzyme in this 0.1 M MES/0.5 M NaCl mobile phase. On the other hand, the residual activity associated with the dimeric and monomeric forms of the enzyme may have been stabilized by the co-eluting BSA dimer and monomer. PEPC has been previously reported to be effectively stabilized by the presence of BSA (Hatch & Oliver, 1978).

In contrast to the above results with 0.5 M NaCl in the incubation buffer, the size-exclusion chromatography studies of PEPC in the presence of 0.5 M NH_4OAc indicated that this salt was less destabilizing toward PEPC at 50 °C with a greater abundance of the active tetrameric form of PEPC present (Figure 5). The major activity peak, however, was broad, suggesting the possibility of a range of conformational and multimer−monomer states of the enzyme under these conditions. The UV absorbance and activity profiles indicate that the dimer of PEPC was also present with a minor peak of enzyme activity eluting with an apparent molecular mass of ~120 kDa, corresponding to the PEPC monomer. The elution profile shows that NH_4OAC has a greater tendency than NaCl to stabilize PEPC in the tetrameric form, although the enzyme can still dissociate into its lower molecular mass subunits. Size-exclusion chromatographic experiments were also carried out with PEPC in the presence of salts determined to be moderate stabilizers at 50 °C, including 0.5 M KOAc and 0.5 M NaOAc, and salts which maximally stabilized PEPC at 52 °C, including 2.5 M NaOAc and 0.8 M NaGlutamate (Table 7). A representative chromatographic elution profile for PEPC incubated in the presence of 0.5 M KOAc indicated that under these conditions PEPC exists almost exclusively in the active tetrameric form (Figure 6). Similar elution profiles were obtained for PEPC incubated in the presence of 0.5 M NaOAc, 2.5 M NaOAc, and 0.8 M NaGlutamate.

DISCUSSION

The effects of salts on the stability of proteins in aqueous solution relate in large measure to the extent by which their

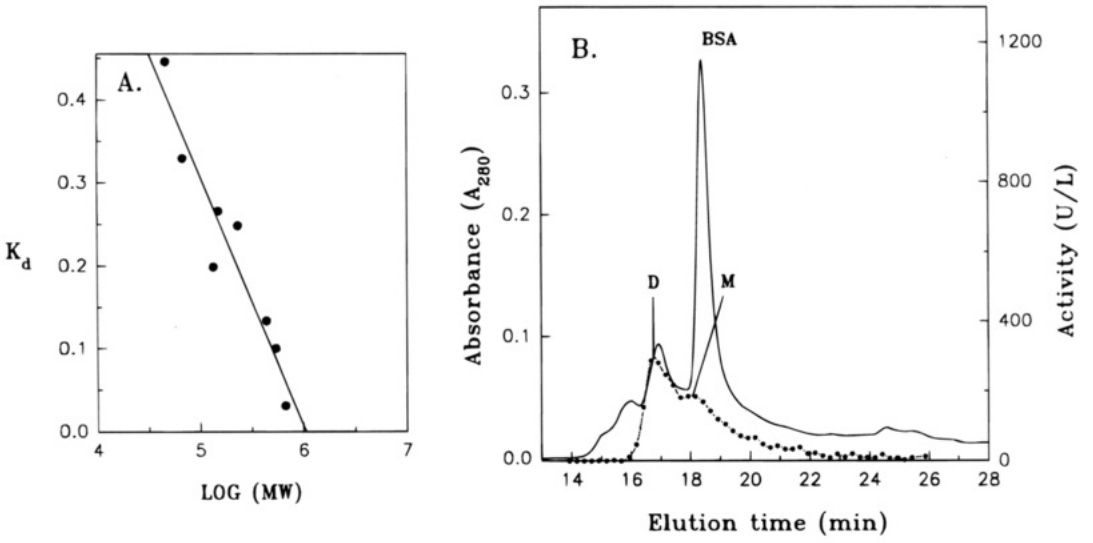

FIGURE 3: (A) Molecular weight calibration profile for the DuPont GF-250 column, with 0.5 M NaCl/0.1 M MES (pH 6.70) as the mobile phase. See Materials and Methods for molecular weight standards. (B) Absorbance (—) and activity (●) profiles for PEPC, eluted from the DuPont GF-250 with 0.5 M NaCl/0.1 M MES (pH 6.70). Letter code: D = PEPC dimer; M = PEPC monomer; BSA = bovine serum albumin.

Table 7: Molecular Mass Estimations (Kilodaltons) for PEPC Peaks Eluted from the DuPont GF-250 Column with Various Mobile Phases Containing 0.1 M MES (pH 6.70)

	PEPC activity in peak profiles[a]			protein absorbance profile maxima (A_{280})[b]		
mobile phase	PEPC tetramer	PEPC dimer	PEPC monomer	PEPC tetraner	BSA/PEPC dimer	BSA/PEPC monomer
0.5 M NaCl	N/D[c]	229	110	389 ± 18	214 ± 8	89 ± 2
0.5 M NH_4OAc	403	N/E[d]	120.2	403 ± 7	254 ± 21	107 ± 9
0.5 M NaOAc	372	N/D	N/D	373 ± 8	229 ± 16	96 ± 12
0.5 M KOAc	452	N/D	N/D	447 ± 31	257 ± 25	105 ± 10
2.5 M NaOAc	403	N/D	N/D	394 ± 14	240 ± 29	91 ± 4
0.8 M NaGlutamate	457	N/D	N/D	452 ± 30	292 ± 17	115 ± 1

[a] Molecular mass estimates were determined from the positions of peak maximal activity of the chromatographic runs. [b] Mean peak molecular mass estimates (±2 standard deviation units error) were calculated from the UV absorbance maxima of the elution profiles of replicate chromatographic runs. [c] N/D = activity peak not detected. [d] NE = active peak elution maximum could not be estimated.

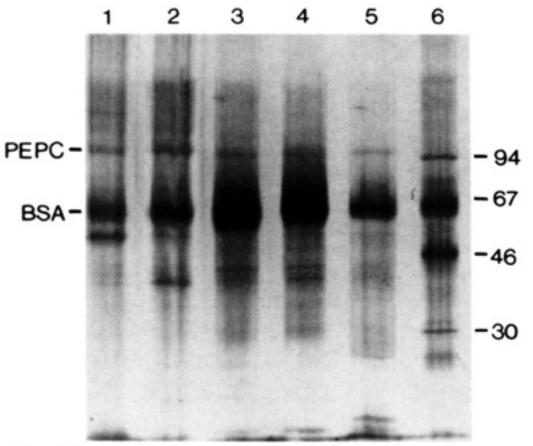

FIGURE 4: SDS−PAGE of PEPC fractions eluted from the DuPont GF-250 with 0.5 M NaCl. Fractions were diluted 1:1 with SDS buffer. Sample load/lane: 1 μL. Lanes: 1, fractions eluting between 15.8 and 16.1 min (376−447 kDa); 2, fractions eluting between 16.8 and 17.1 min (200−235 kDa); 3, fractions eluting between 18.1 and 18.3 min (94−110 kDa); 4, fractions eluting between 18.3 and 18.6 min (81−94 kDa); 5, fractions eluting between 19.6 and 19.8 min (<BSA); 6, protein standards (20 ng/μL). See Materials and Methods for protein standards.

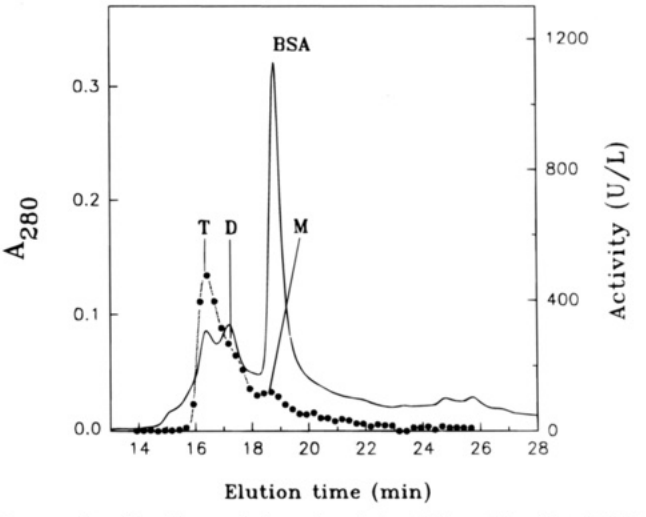

FIGURE 5: Absorbance (—) and activity (●) profiles for PEPC eluted from the DuPont GF-250 column with 0.5 M NH_4OAc/0.1 M MES, (pH 6.70). Letter code: T = PEPC tetramer; D = PEPC dimer; M = PEPC monomer; BSA = bovine serum albumin.

constituent ions can interact with the dipolar water molecules of the solution and thus affect water structure. The water-structuring effectiveness of an ion is closely related to its charge density and follows the order $PO_4^{3-} > F^- > SO_4^{2-} > Cl^- > Br^- > I^- > NO_3^- > BrO_3^- > ClO_4^-$ for anions while for cations the order of water-structuring effectiveness is $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} > Li^+ > Na^+ > K^+ > NH_4^+ > Rb^+ > Cs^+$ (von Hippel & Schleich, 1969; Burgess, 1988). Ions above Br^- and Na^+ in their respective series confer an increasingly ordered structure to water (the so-called kosmotropic effect), while ions below Cl^- and Li^+ in their respective series induce an increasing loss in the

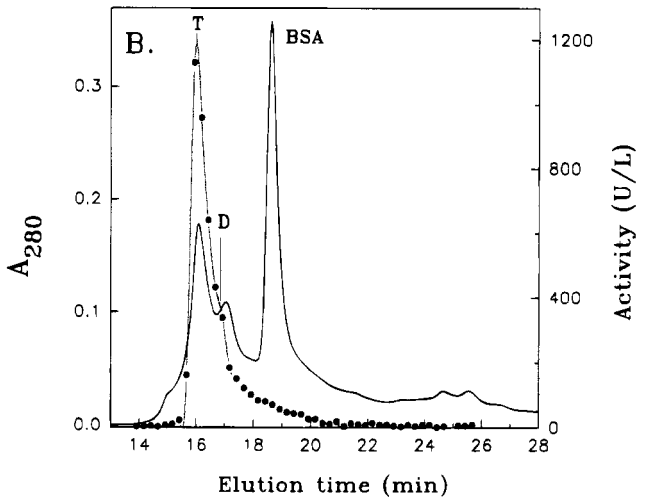

FIGURE 6: Absorbance (—) and activity (●) profiles for PEPC eluted from the DuPont GF-250 with 0.5 M KOAc/0.1 M MES (pH 6.70). See Figure 5 for letter descriptions.

structural order of the dipolar water molecules (a chaotropic effect). The above anion series correlates closely with data compiled for the Hofmeister series of anions in terms of their ability to function as "salting out" or stabilizing reagents with proteins (Hofmeister, 1888; Collins & Washabaugh, 1985), where the series typically follows the order citrate^{3-} > PO_4^{3-}/HPO_4^{2-}, SO_4^{2-} > OAc^-, F^- > Cl^- > Br^- > I^- > ClO_4^-.

The salting out and stabilization effects of salts on proteins have been attributed to the promotion of intramolecular and intermolecular hydrophobic interactions involving the protein molecules due to the water-structuring effects of the ions (von Hippel & Wong, 1965; Hatefi & Hanstein, 1969). This phenomenon has been investigated by Horvath and co-workers (Melander & Horvath, 1977; Melander et al., 1985), who demonstrated that the predominant salting out effect of salts is related to the ability of any given salt to increase the molal surface tension increment of a solution, an observation in agreement with the solvophobic theory proposed by Sinanoğlu and co-workers (Sinanoğlu & Abdulnur, 1965; Halicioğlu & Sinanoğlu, 1969; Sinanoğlu, 1980). According to these solvophobic concepts, salts which increase the structure, and more specifically the surface tension, of a solution will increase the energy required to expand and extend the dimensions of the solvent cavity in which a protein resides. Anions or cations which induce salting out effects by increasing the surface tension properties of a solvent are therefore expected to increase the stability of globular proteins in solution. Kella and Kinsella (1988) have correlated the salt surface tension effect with the stabilization of β-lactoglobulin, while Hearn and co-workers (Hearn et al., 1985; Hearn & Aguilar, 1988) have shown that related effects operate with solvent destabilization of the tertiary structure of polypeptides or proteins, resulting from the lowering of the surface tension of the solution with aqueous–organic solvent mixtures.

In addition to their effects on solvent structure, salts can also affect the tertiary or quaternary structure of a protein in solution by promoting solubilization (the "salting in" effect). This effect involves the preferential interaction of salt components with the protein (Bello, 1963; Arakawa & Timasheff, 1982, 1984a,b, 1985; Arakawa et al., 1990; Schellman, 1990), and usually results in the destabilization of the macromolecule's native structure. Conversely, stabilizing salts preferentially are excluded from the protein for more favourable interactions with the bulk solvent phase (Arakawa & Timasheff, 1982, 1984a,b; Arakawa, 1986; Timasheff & Arakawa, 1989; Schellman, 1990). There is strong experimental support for the concept that the mechanism of destabilization of proteins by some salts involves ion binding to polar groups of the protein, in particular to the dipoles of the peptide bond and the side-chain amide groups (Bello et al., 1956; Mandelkern & Stewart, 1964; Robinson & Jencks, 1965; Schrier & Schrier, 1967; von Hippel et al., 1973; Arakawa & Timasheff, 1985), rather than as a result of nonspecific effects of the salt on the structure of water (von Hippel & Wong, 1965). The stability of a protein in solution in the presence of salts can therefore be generally considered to represent a balance between these two predominant but opposing mechanisms, one resulting from the surface tension effects of the ions on the solvent molecules and the other from the binding of ions to polar groups of the protein.

Thermal Inactivation of PEPC. As evident from the results presented above, the stabilization and destabilization of PEPC activity by the various salts examined in this study are consistent with the interplay between surface tension effects and ion binding effects. For example, the extent of retention of the catalytic activity of PEPC after the thermal treatment depends both on the nature of the salt used and on the salt concentration. From the experimental results shown in Tables 1–5 and Figures 1 and 2, a general pattern of stabilization effectiveness can be recognized for the various anions and cations examined. For the anions, the stabilization effectiveness (i.e., the protective ability to reduce the extent of thermal inactivation) was in the order citrate^{3-}/citrate^{2-} > SO_4^{2-} ≥ HPO_4^{2-}/$H_2PO_4^-$ > OAc^- > F^- > Cl^- > Br^-. This result is consistent with the Hofmeister series, indicating that the water-structuring effects of the anions are significant in determining the extent to which the thermal inactivation of PEPC can be protected by an individual anion. Consequently, it can be concluded from the results on thermal inactivation that kosmotropic anions increasingly stabilized PEPC, while the anions Cl^- and the chaotrope Br^- increasingly destabilized the enzyme. Moreover, it can be noted that the effects of these anions on PEPC activity can change markedly with concentration.

In contrast, the stabilization effectiveness of the tested cations showed considerable deviation from the order found in the water-structuring series of cations, the order being Rb^+ ≥ K^+, Na^+, Cs^+, $(CH_3)_4N^+$, NH_4^+ > Li^+ for PEPC "protection". The extent of stabilization of PEPC was not significantly different for Rb^+, K^+, Na^+, and Cs^+; however, $(CH_3)_4N^+$, NH_4^+, and Li^+ destabilized the enzyme. An exception to the rule was noted, however, when $(CH_3)_4N^+$ was paired with Cl^-. The more pronounced destabilizing effect of cations (particularly those highly placed in the water-structuring series of cations) compared to anions is consistent with the observation that the C=O groups of the peptide bonds or side-chain amides represent strong hydrogen bond acceptors, while the NH groups of the peptide bonds or side-chain amides are weak donors (Singer, 1962; Ramakrishnan & Prasad, 1971; Alagona et al., 1973; Baker & Hubbard, 1984). The low correlation observed between the order of cations for the maintenance of PEPC activity under thermal conditions and the water-structuring series of cations also indicates that solvent-ordering effects mediated by these

cations are not the predominant contributor toward the stabilization of PEPC at elevated temperatures but rather that cation−protein binding interactions are influential.

(i) Salt−PEPC Protein Interactions. The destabilization of PEPC by some of the salts studied at 0.5 M was associated with the dissociation of the enzyme into its dimeric and monomeric forms, as apparent from the elution profiles obtained by HPSEC of PEPC following incubation and elution in the presence of 0.5 M NaCl (Figure 3B) and 0.5 M NH_4OAc (Figure 5). These results correlate with the findings of other investigators, whereby the presence of 200−400 mM NaCl has been observed to promote the dissociation of PEPC into the dimeric and monomeric forms in both dilution studies (McNaughton et al., 1989) and at pH values distant from the enzyme's pH optimum (Wagner et al., 1987). Results from our associated investigations involving kinetic studies on PEPC inactivation under analogous thermal conditions (W. A. Jensen, J. McD. Armstrong, and M. T. W. Hearn, unpublished results) have confirmed that the salts of Br^-, Cl^-, OAc^- SO_4^{2-}, HPO_4^{2-}, and NaGlutamate are not involved in specific ion−protein interactions which affect the activity of PEPC. However, potassium citrate specifically inhibited PEPC in a concentration-dependent manner, indicating that the stability of the enzyme may be affected by the interaction of the citrate anion with the catalytic or allosteric sites of the protein. The destabilization and solubilization of PEPC by salts low in the Hofmeister series therefore appear to result from general salt−protein interactions, mediated by ion−peptide dipole and ion−charged amino acid residue binding effects, rather than by ion interaction with specific, high-affinity binding sites on the protein. In considering ion−peptide dipole interactions as the major source of PEPC destabilization by salts, the increased interaction of the polypeptide backbone dipoles and the polar amino acid side chain groups with the solvated ions will reduce the free energy of cavity formation (ΔG_{cav}) (Sinanoğlu & Abdulnur, 1965; Sinanoğlu, 1968; Hearn et al., 1985; Hearn & Aguilar, 1988). This reduction in ΔG_{cav} will favor an expansion of the macromolecule and an increase in the surface area of the cavity: i.e., on a macroscopic scale the protein will begin to unfold, and multimeric forms of PEPC will begin to dissociate into the constituent subunits.

(ii) Salt−Water Interactions. Solvent-mediated effects on protein stability become increasingly noticeable at high salt concentrations, where the structure and surface tension of the solvent are significantly changed. Salts of polyvalent anions with high charge densities would be expected, because of their relative effectiveness in elevating the surface tension of a solution, to promote at lower concentrations the maximum stabilization of PEPC activity as compared to salts of monovalent anions. Indeed, this effect was observed for the divalent anions with PEPC (see Figure 2). In addition, the aggregation and salting out of PEPC was induced more effectively by salts high in the Hofmeister series (Table 6). With such salts, e.g., HPO_4^{2-} or SO_4^{2-}, the ion−peptide dipole interactions are negligible but the surface tension effects are greatest. The stabilization and salting out effects noted with PEPC and the salts of anions high in the Hofmeister series correlate with the concepts of the solvophobic theory whereby an increase in cavity free energy will induce stronger intersubunit hydrophobic interactions, resulting in the maintenance of the tetrameric structure of PEPC. With these salts at very high concentrations, e.g., >2 M, aggregation and precipitation of PEPC from solution can result due to the high surface tension values. An exception to this behavior in terms of the general order of the salting out series was noted with the effects of potassium citrate on PEPC solubility, with no salting out effects observed at concentrations up to 1.3 M potassium citrate.

Sodium glutamate elicited a maximum stabilization of PEPC at concentrations which were far below those of the other monovalent anionic salts. This result is also in agreement with solvophobic considerations and indicates that glutamate ions can interact effectively with the dipoles of water to raise the surface tension of the solution, which favors the folded, tetrameric state of the enzyme. Sodium glutamate is, in addition, known to be preferentially excluded from the domain of proteins such as BSA, lysozyme, β-lactoglobulin, and tubulin (Arakawa & Timasheff, 1984c). Consequently, a competitive weakening of the protein cavity effects by a reduction in ΔG_{int} due to the binding of glutamate ions to the protein domain is likely to be negligible in the case of PEPC. This conclusion is in accordance with the results of Arakawa and Timasheff (1984c) with lysozyme or β-lactoglobulin, which demonstrated that the effects of this amino acid may generally be mediated through its ability to significantly elevate the surface tension of the solution.

CONCLUSIONS

It is apparent from the present investigations that the stabilization of PEPC by various salts at elevated temperatures is a consequence of the interplay of two opposing mechanisms. On the one hand, the capability of the salts containing chaotropic anions, in particular, to interact with the polypeptide backbone and side-chain amide dipoles of the protein results in the destabilization of the enzyme. This form of interaction weakens the cavity effect and encourages the dissociation of PEPC into its subunits. On the other hand, the propensity for salts containing anions high in the Hofmeister series to interact strongly with the dipoles of water, thereby increasing the positive molal surface tension increment conferred on the solution, tends to predominate over the weaker interactions of these salts with the dipoles of the protein. In accord with the solvophobic theory, the rise in surface tension of the solution at higher salt concentrations encourages a reduction in the unfavorable surface area of contact between the protein and the solvent, a consequent increase in intramolecular and intermolecular hydrophobic interactions, and enhanced stabilization of a compact, tetrameric conformation for PEPC. Above the salt concentration for the maximal stabilization of the protein, however, this latter effect results in the inactivation of PEPC, presumably due to the reinforcement of these additional hydrophobic interactions. This finding indicates that PEPC not only is destabilized at low salt concentrations by more favorable solvation (which leads to subunit dissociation) but also can be destabilized as a consequence of the desolvation of the enzyme at high salt concentrations. Importantly, the present studies have shown that conditions can be developed using appropriate anion and cation combinations to stabilize PEPC in solution at elevated temperatures for considerable periods of time. Implementation of such stabilization conditions is relevant not only to the use of PEPC as a clinical diagnostic enzyme but also with other multimeric

proteins which readily dissociate to their subunits on storage in solution.

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