Articles

Structural Properties of UMP-Kinase from *Escherichia coli*: Modulation of Protein Solubility by pH and UTP†

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ABSTRACT: UMP-kinase from *Escherichia coli*, unlike the analogous enzyme from eukaryotic organisms, is an oligomeric protein subjected to complex regulatory mechanisms in which UTP and GTP act as allosteric effectors. While the enzyme has an unusually low solubility at neutral pH (≤0.1 mg of protein/mL), its solubility increases markedly above pH 8 and below pH 4. Furthermore, the solubility of the bacterial UMP-kinase at neutral pH is greatly enhanced in the presence of Mg-free UTP. Thermal denaturation experiments have demonstrated that UTP also increases the stability of the protein. Fourier-transform infrared spectroscopy and circular dichroism show that the secondary structure of the protein is the same at neutral and at alkaline pH. These data indicate that variations in enzyme solubility must be related to subtle changes in the tertiary and/or quaternary structure which modulate the exposure of hydrophobic surfaces in the protein molecule. A variant of UMP-kinase, obtained by site-directed mutagenesis ($Asp_{159}Asn$), which is similar to the wild-type enzyme in its stability and kinetic properties, has a much increased water solubility (>5 mg protein/mL) even at neutral pH. This suggests that salt bridges may be involved in the equilibrium between the soluble and aggregated forms of the wild-type enzyme, and that conformational changes induced upon binding of UTP increase the protein solubility by disrupting these salt bridges.

Nucleoside monophosphate kinases (NMP-kinase)[1] are ubiquitous enzymes which play a major role in the energy metabolism, in the synthesis of nucleic acid precursors, and in the synthesis of various nucleotidyl-containing intermediates (Anderson, 1973; Neuhard & Nygaard, 1987). Believed to derive from a common ancestor, they exhibit a relatively narrow specificity for the nucleoside monophosphate molecule, the acceptor of the phosphoryl group. Thus, most cells contain separate AMP-, GMP-, UMP-, and CMP-kinases which act on the ribo- and deoxyribomonophosphates of the purine and pyrimidine nucleobases. Generally, these enzymes are small monomeric proteins with significant sequence homology and closely related three-dimensional structures, as inferred from X-ray crystallographic studies (Liljelund et al., 1989; Wiesmüller et al., 1990; Konrad, 1992; Müller-Dieckmann & Schulz, 1994).

The UMP-kinase from *Escherichia coli*, whose gene was recently cloned by several groups (Yamanaka et al., 1992; Smallshaw & Kelln, 1992; Serina et al., 1995), deviates from this paradigm. The protein does not share any sequence similarity with other known NMP-kinases, has an oligomeric structure, and is subjected to complex regulatory mechanisms in which UTP and GTP act as allosteric effectors (Serina et al., 1995).

An intriguing and unique property of UMP-kinase from *E. coli* is the very low solubility at neutral pH (≤0.1 mg/mL). UTP and/or alkaline pH increase the solubility of the bacterial enzyme significantly. To understand the relationship between the structure of this enzyme and its unusual characteristics, we undertook a systematic study of UMP-kinase using a variety of physicochemical methods. Our data suggest that formation of salt bridges between charged amino acid residues on each oligomer promotes exposure of hydrophobic surfaces, leading to extensive aggregation of the protein at neutral pH. We surmise that upon binding of

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[1] Abbreviations: ANS, 8-anilino-1-naphthalene sulfonic acid; FT-IR, Fourier transform infrared spectroscopy; GMP-PNP, guanylyl imido diphosphate; IPTG, isopropyl β-D-thiogalactoside; NDP-kinase, nucleoside diphosphate kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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UTP, conformational changes occuring in a concerted manner disrupt these salt bridges, resulting in the solubilization of the UMP-kinase.

MATERIALS AND METHODS

Chemicals. Adenine nucleotides, restriction enzymes, T4DNA ligase, and coupling enzymes were from Boehringer Mannheim. T7 DNA polymerase and the four deoxynucleoside triphosphates used in sequencing reactions were from Pharmacia. Oligonucleotides were synthesized according to the phosphoamidinate method using a commercial DNA synthesizer (Cyclone TM Biosearch). NDP-kinase from *Dictyostelium discoideum* (2000 units/mg of protein) was kindly provided by M. Véron.

Bacterial Strains, Plasmids, Growth Conditions, and DNA Manipulations. Strain BLI5 used for overexpression of the *pyrH* gene encoding the UMP-kinase was derived from BL21 (DE3) (Novagen Inc.). The strain expresses the *lacI* gene on plasmid pDIA17 and the T7RNA polymerase gene on the chromosome. Cultures were performed in 2YT medium (Sambrook et al., 1989) supplemented with 100 μg/mL ampicillin and 30 μg/mL chloramphenicol. When OD_{600} reached 1.5, 1 mM IPTG was added to the medium. The bacteria were harvested by centrifugation 3 h after induction. Site-directed mutagenesis was performed on the single-stranded DNA form of phagemid pDIA5418 grown in strain CJ236 in the presence of plasmid pDIA17 and the helper phage M13K07. The Asp (GAT) codon at position 159 was modified to a Asn (AAT) codon using the oligonucleotide 5′-CAGCACCACATTGGCTTCAATTTC-3′. The absence of unwanted mutations during site-directed mutagenesis was verified by the dideoxynucleotide sequencing method (Sanger et al., 1977).

Purification of UMP-Kinase and Activity Assay. Wild-type UMP-kinase from *E. coli*, overproduced in the same bacteria, was purified as described previously (Serina et al., 1995). The $D_{159}N$ variant, overexpressed in *E. coli*, was purified as follows. Bacteria suspended in 50 mM Tris-HCl (pH 7.4) were sonicated, and then the extract was centrifuged at 10000*g* for 30 min. The supernatant was heated at 65 °C for 15 min, and the precipitated protein was removed by centrifugation. The supernatant was applied on a DEAE-Sepharose CL-6B column equilibrated with the same buffer and the enzyme eluted with a linear gradient of NaCl (0−0.3 M) in 50 mM Tris-HCl (pH 7.4). The fractions eluting between 0.12 and 0.17 M NaCl were pooled and concentrated by ultrafiltration using a 10 mL Omegacell (Filtron). A second chromatography was performed on Sephacryl S-300 HR (0.9 × 104 cm) at a flow rate of 5 mL/h. The purified protein concentrated to about 5 mg/mL was kept at +4 °C. The UMP-kinase activity was determined at 30 °C using a coupled spectrophotometric assay (0.5 mL final volume) on an Eppendorf PCP6121 photometer (Blondin et al., 1994). One unit of the enzyme corresponds to 1 μmol of product formed per min. UMP-kinase activity in the presence of GdmCl was assayed by an end-point procedure. The reaction medium (0.2 mL) which contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 4 mM ATP, 2 mM UMP, 1 mM GMP-PNP, 50 mM KCl, and various concentrations of GdmCl (between 0 and 2 M) was incubated with UMP-kinase for 2−10 min. Fifty microliters of this mixture was subsequently added to 0.45 mL of a medium containing 50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 50 mM KCl, 1 mM phosphoenolpyruvate, 0.25 mM NADH, and 2 units each of lactate dehydrogenase and NDP-kinase. The absorbance at 334 nm was determined, 2 units of PK was added to this mixture and the decrease in absorbance measured after 1 min. The above assay, which requires the coupling enzymes only in its final step, was highly reproducible (±5% error).

Fluorescence Measurements. Fluorescence experiments were performed on a Perkin-Elmer LS-5B luminescence spectrometer thermostated at 25 °C. Emission spectra of UMP-kinase (λ_{exc} = 295 nm; band width = 5 nm) were recorded from 305 to 400 nm. Binding of nucleotides to UMP-kinase was followed by fluorescence enhancement at 330 nm.

Infrared and CD Spectroscopy. Circular dichroism was measured with a J-710 spectropolarimeter (Jasco, Japan) at room temperature (22 ± 2 °C). The band width was 2 nm, and spectra were usually averaged over 10 scans to improve the signal to noise ratio. CD spectra were measured in 0.1 cm path length cell, at a protein concentration of about 0.1 mg/mL. Estimation of protein secondary structure from CD spectra was obtained by curve fitting, using the software provided by Jasco. As reference spectra of α-helix, β-sheet, β-turn, and random coil structures, the data reported by Yang et al. (1986) were used.

Infrared spectra were recorded on a Digilab FTS-40A FT-IR spectrometer equipped with a liquid nitrogen cooled mercury−cadmium−telluride detector and continuously purged with dry air. Samples were suspended in 10 mM sodium cacodylate buffer at a protein concentration of ∼10 mg/mL; the pH was adjusted to the required value with NaOH or HCl. The clear protein solutions (at pH 9), or the suspension-like samples of UMP-kinase at pH 7, were placed between two CaF_2 windows separated by 6 μm. Concentrated UTP and GTP solutions were added to the protein solutions to yield nucleotide protein mixtures of 2:1 molar ratio. For each sample, 512 interferograms were co-added and Fourier-transformed to generate a spectrum with a nominal resolution of 2 cm^{-1}. Protein spectra were obtained by subtracting the aqueous buffer spectrum from the spectrum of the corresponding protein. Spectral contributions from residual water vapor were eliminated using a set of water vapor spectra measured under identical conditions. The subtraction factor was varied until the second derivative of the absorption region above 1720 cm^{-1} was featureless, thereby avoiding artificial bands/or incorrect band positions in the amide I and amide II region of the protein spectrum (Jackson & Mantsch, 1995). The final unsmoothed protein spectra were used for further analysis. Fourier self-deconvolution was performed as described previously (Mantsch et al., 1988) using a half bandwidth of 16 cm^{-1} and a band-narrowing factor k = 2.0. Deconvolved infrared spectra were fitted with Gaussian band profiles using software developed in-house.

Analytical Centrifugation. Centrifugation was performed at 20 °C on a Beckman Optima XLA ultracentrifuge using a AN 60 Ti titanium four-hole rotor and a cell with two-channel 12-mm pathlength centerpieces. The partial specific volume for UMP-kinase (0.746 cm^3 g^{-1} at 20 °C) was computed from the amino acid composition by use of the additivity rule and published values for individual amino acid residues (Cohn & Edsall, 1943). The density of each buffer was calculated.

Sedimentation Equilibrium Analysis. Sedimentation equilibrium experiments were carried out using a loading concentration of 0.3 mg/mL (12 μM) in 50 mM sodium borate (pH 9). Three rotor speeds (6000, 10 000, 20 000 rpm) were used to ensure a clear separation of various oligomeric forms at equilibrium. Radial scans of absorbance at 280 nm were taken, and samples were judged to be at equilibrium by the absence of systematic deviations in overlayed successive scans and when a constant average molecular mass was obtained in the plots of MW versus centrifugation time. Concentration distributions were analyzed by fitting appropriate mathematical models using the Optima XL-A Data Analysis software package supplied by Beckman. The experiment was also run in the presence of 1 and 3 M GdmCl.

Sedimentation Velocity Analysis. Sample volumes of 400 μL in 50 mM acetate/acetic acid (pH 6) Tris-HCl (pH 7.4) or sodium borate (pH 9) were centrifuged at 50 000 rpm, and radial scans of absorbance at 280 nm were taken at 5 min intervals. Data analysis was performed using the computer programs XLAVEL and XLQ-VELOC supplied by Beckman and Svedberg (Philo, 1994). Hydrodynamic parameters of UMP-kinase molecular species were evaluated using the program AXIAL provided by Les Holladay. The molecular mass of various species of UMP-kinase (M_2) was calculated using the relation $S_1/S_2 = [M_1/M_2]^{2/3}$ where S_1 and M_1 represent the sedimentation coefficient and the molecular mass of bovine serum albumin.

Other Analytical Procedures. Protein concentration was measured according to Bradford (1976) or by amino acid analysis on a Beckman system 6300 high-performance analyzer after 6 N HCl hydrolysis for 22 h at 110 °C. SDS−polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The thermal stability of UMP-kinase in the absence and presence of various ligands was investigated by high-sensitivity differential scanning calorimetry using a Microcal MC-2D instrument and a protein concentration of approximately 0.8−1 mg/mL. The buffer used for calorimetry experiments was 100 mM borate, pH 8.5. Prior to the experiments, protein solutions were dialyzed against the same batch of the buffer and degassed. A portion of the dialysis buffer was used in the reference cell. The calorimetric traces were filtered and analyzed using the software provided by Microcal Corp. T_m values were defined as the maxima of the excess heat capacity versus temperature functions.

RESULTS

Effect of pH and of Nucleotides on the Solubility of UMP-Kinase. When *E. coli* overexpressing the *pyrH* gene was disrupted by sonication in 50 mM Tris-HCl buffer (pH 7.4), over 95% of the enzyme activity was recovered in the pellet after 30 min of centrifugation at 10000*g*. When the same protocol was used with 100 mM borate (pH 9) or by supplementing the Tris-HCl buffer with 1 mM UTP, the UMP-kinase was recovered mainly in the supernatant (Figure 1A). Solubilization of the protein by alkaline pH or by addition of UTP can be reversed by lowering the pH of the supernatant or by adding an excess over the nucleotide concentration of Mg^{2+} (Figure 1B). These above solubility properties provide a convenient way for enzyme purification without the use of conventional chromatographic procedures.

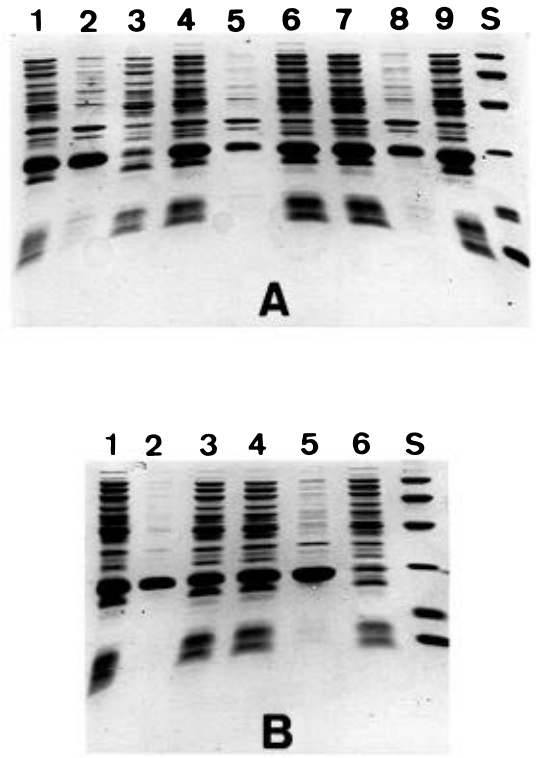

FIGURE 1: Effect of alkaline pH and of UTP on the solubility of UMP-kinase from *E. coli*. (A) Bacteria overproducing wild-type UMP-kinase from *E. coli* were divided into three equal portions and resuspended in 50 mM Tris-HCl (pH 7.4) (lanes 1−3), 100 mM sodium borate (pH 9) (lanes 4−6), or 50 mM Tris-HCl (pH 7.4) supplemented with 1 mM UTP (lanes 7−9). The bacteria were sonicated (six pulses of 1 min at 100 W and 20 kHz) and then centrifuged at 10000*g* for 30 min. The supernatants were saved and the pellets resuspended in the original volume of each buffer system. Lanes 1, 4, and 7 represent total bacterial extract each corresponding to 10 μg of protein; lanes 2, 5, and 8 represent the resuspended pellets while lanes 3, 6, and 9 represent the corresponding supernatants. The amount of protein found in lanes 2 + 3, 5 + 6, and 8 + 9 corresponds to that in lanes 1, 4, and 7, respectively (i.e., 10 μg each). (B) Reversibility of UMP-kinase solubilization by alkaline pH and by UTP. Cell-free extracts containing UMP-kinase from *E. coli* solubilized by alkaline pH (lanes 1−3) or by 1 mM UTP (lanes 4−6) were treated with 2 N HCl or with 1 M $MgCl_2$ to lower the pH value to ~7 or to attain a Mg^{2+} concentration of 10 mM. After 2 h of storage at +4 °C, the extracts were centrifuged at 10000*g* for 30 min. The pellets were resuspended to the original volume of extracts with 100 mM sodium borate (pH 9) or with 50 mM Tris-HCl (pH 7.4) supplemented with 1 mM UTP and electrophoresed on SDS−PAGE. Lanes 1 and 4, cell-free extracts (~10 μg of protein); lanes 2 and 5, pellet after centrifugation at 10000*g*; lanes 3 and 6, supernatants after centrifugation at 10000*g*. Lane S in panels A and B corresponds to standard proteins, from top to bottom: (a) phosphorylase a (94 000); (b) bovine serum albumin (68 000); (c) ovalbumin (43 000); (d) carbonic anhydrase (30 000); (e) soybean trypsin inhibitor (20 100); (f) lysozyme (14 400).

The purified UMP-kinase was stored at +4 °C as a suspension in 50 mM Tris-HCl (pH 7.4). Under these conditions, the concentration of the soluble form is about 0.1 mg/mL, i.e., 4 μM in terms of the protein monomer. For routine enzymatic assays or for spectroscopic studies which required low concentrations of UMP-kinase (less than 4 μM), the suspension was diluted directly with 50 mM Tris-HCl (pH 7.4). Measurement of the activity at various intervals of time showed that the enzyme retained its full activity even after months of storage in the aggregated form.

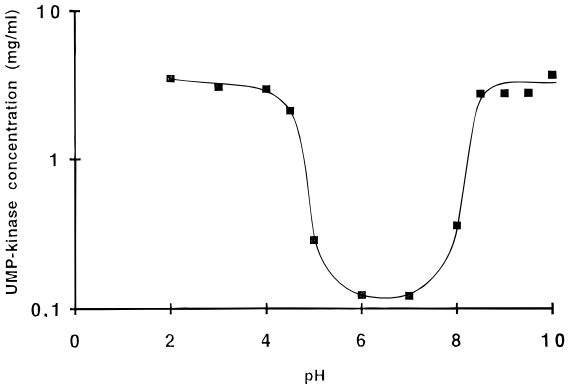

FIGURE 2: Solubility of UMP-kinase as a function of pH. UMP-kinase stored as supension in 50 mM Tris-HCl (pH 7.4) at approximately 6 mg of protein/mL was centrifuged for 10 min at 10000*g*. The pellet was resuspended in the same volume of various buffers at 50 mM having pH values between 2 and 10. After 1 h of storage at room temperature, the samples were centrifuged for 10 min at 10000*g*, and the supernatant was used for protein concentration determination and activity measurements. The following buffers were used: glycocol/HCl (pH 2 and 3); sodium acetate/acetic acid (pH 4.5−6); KP_i/K_2P_i (pH 6, 7, and 8); Tris-HCl (pH 7.4, 8, 8.5, and 9); borate (pH 8, 8.5, and 9); glycocol/NaOH (pH 9.5 and 10).

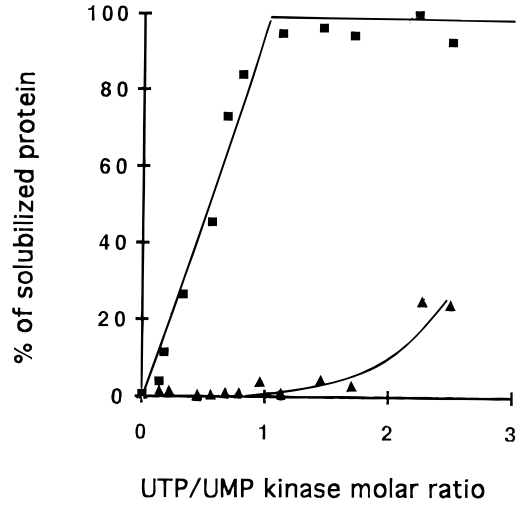

FIGURE 3: Dependence of UMP-kinase solubility on UTP concentration. UMP-kinase stored as suspension in 50 mM Tris-HCl (pH 7.4) at approximately 6 mg of protein/mL was supplemented with various concentrations of UTP in the absence (■) or in the presence of 2 mM $MgCl_2$ (▲). After 1 h of incubation at room temperature, the samples were centrifuged for 10 min at 10000*g*, and the supernatant was used for protein concentration determination and activity measurements. The molecular mass of UMP-kinase monomer was considered as 25.8 kDa.

A more detailed analysis of the pH effect showed that the solubility of UMP-kinase is minimal between pH 5.5 and 7.5, and that below pH 4 or above pH 9 the solubility attains its maximum (Figure 2). Furthermore, determination of the enzyme activity at pH 7.4 after dilution of the protein in various buffers over the pH range 2−10 indicated that the UMP kinase was stable between pH 4 and 10, while below pH 4 it was irreversibly inactivated.

The solubilization of UMP-kinase by UTP required 1 mol of nucleotide per subunit (Figure 3). By simultaneously varying the concentration of UTP and of Mg^{2+} and then computing the concentration of Mg-bound UTP and Mg-free UTP, we found that, unlike free UTP, the metal-complexed nucleotide was unable to increase the solubility

Table 1: Effect of Different Ligands on the Midpoint Denaturation Temperature of UMP-Kinase

ligand	T_m (°C)
none	61.6
UTP, 0.1 mM	74.6
UTP, 1 mM	82.4
UTP, 5 mM	86.5
GTP, 0.1 mM	62.7
GTP, 1 mM	65.8
GTP, 5 mM	69.8
ATP, 1 mM	63.7
UMP, 1 mM	75.6

of UMP-kinase. The dissociation constant (K_d) of Mg^{2+}/ATP or Mg^{2+}/UTP complexes are identical, and, under our experimental conditions, they are approximately 0.1 mM (Bârzu et al., 1976). Of all other nucleotides tested, only UDP and dUTP had an effect similar to that of UTP on the solubility properties of the enzyme.

Temperature and GdmCl Denaturation of UMP-Kinase. The thermal stability of UMP-kinase alone or in the presence of various ligands was tested by differential scanning calorimetry. Denaturation of bacterial enzyme by heat was irreversible: after cooling from the first run, no transition could be detected in the second heating cycle. Furthermore, the calorimetric traces were somewhat scan-rate dependent (data not shown), indicating that the denaturation process is, at least to some extent, kinetically controlled. Extensive aggregation of the protein occurred during thermal denaturation, resulting in a small exothermic effect and significant baseline abberations at posttransitional temperatures. This precluded the interpretation of calorimetric data in terms of equilibrium thermodynamics (Sanchez-Ruiz et al., 1988; Freire et al., 1990). Nonetheless, the midpoint denaturation temperatures (T_m values) at a given scan rate (60 °C/h) were used as an empirical parameter to assess thermal stability of the enzyme in the presence of various ligands (Table 1). The nucleotides ATP and GTP have only a relatively small effect on the stability of the enzyme, whereas UMP and UTP produce a marked increase in T_m.

The UMP-kinase from *E. coli* was stable in GdmCl up to approximately 1 M. Half-inactivation of the enzyme occurred at 1.8 M GdmCl (Figure 4A) and was accompanied by a red-shift in the maximum of tryptophan fluorescence (Figure 4B) and a decrease in ellipticity at 222 nm (Figure 4C). The enhancing effect of UTP on the intrinsic fluorescence of UMP-kinase was lost at concentrations of GdmCl below 1 M (not shown). This effect may be related to subtle conformational changes in individual monomers since the oligomeric structure and the secondary structure of the protein were not affected (see below).

Spectroscopic Studies. The infrared spectrum of UMP-kinase in H_2O buffer at neutral pH shows two groups of bands: the amide I band (in the range 1620−1690 cm^{-1}) which arises primarily from stretching vibrations of the backbone amide C═O groups, and the amide II band centered at 1550 cm^{-1}. Band narrowing by Fourier self-deconvolution (Figure 5) reveals that the amide I band contour is composed of several component bands, the positions of which provide information concerning protein secondary structure (Surewicz et al., 1993; Jackson & Mantsch, 1995). The band at 1639/1640 cm^{-1} is diagnostic of amide groups in intramolecular β-sheet conformation with relatively weak hydrogen bonds. The amide I bands between

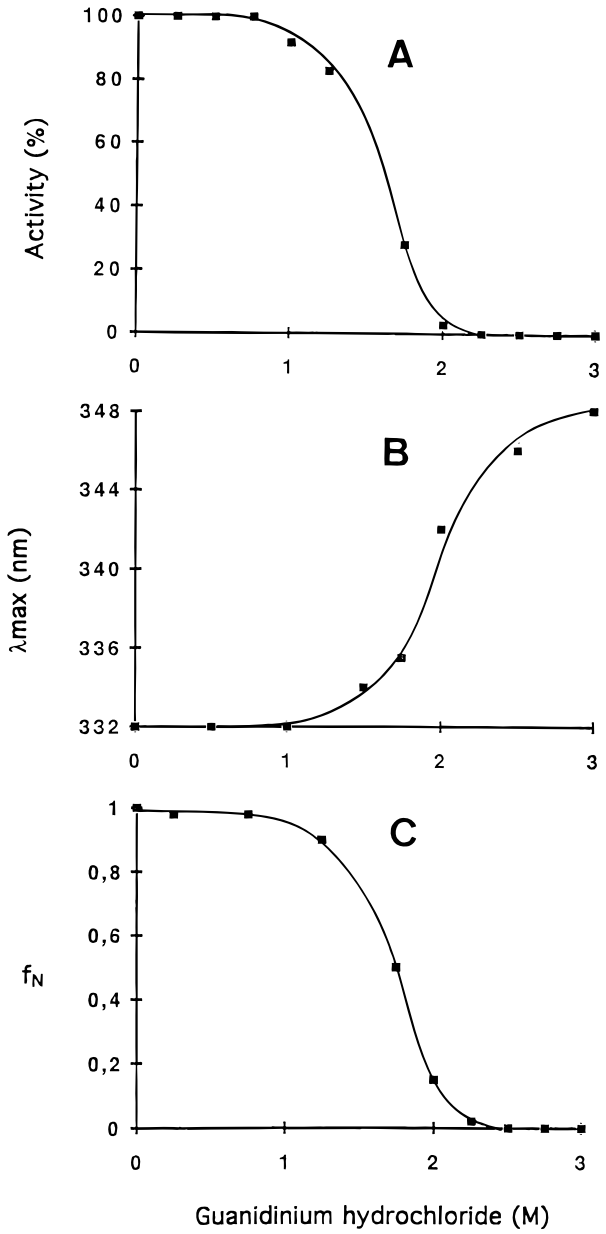

FIGURE 4: Dependence of UMP-kinase activity, maximum of fluorescence intensity and fraction of folded protein on GdmCl concentration. UMP-kinase was diluted in 100 mM sodium borate (pH 8.5) containing various concentrations of GdmCl (between 0.25 and 3 M), at a protein concentration of about 0.1 mg/mL (~4 μM). After 12 h storage at room temperature, each sample was analyzed for activity (A), fluorescence (B), and circular dichroism (C). Enzyme activity was determined by a coupled spectrophotometric assay or by an end-point procedure as described under Experimental Procedures. The fraction of folded protein (f_N) was calculated as $f_N = (\theta-\theta_N)/(\theta_N-\theta_u)$ were θ is the observed ellipticity at 222 nm at some point in the transition, and θ_N and θ_u are the values for the native and unfolded forms, respectively.

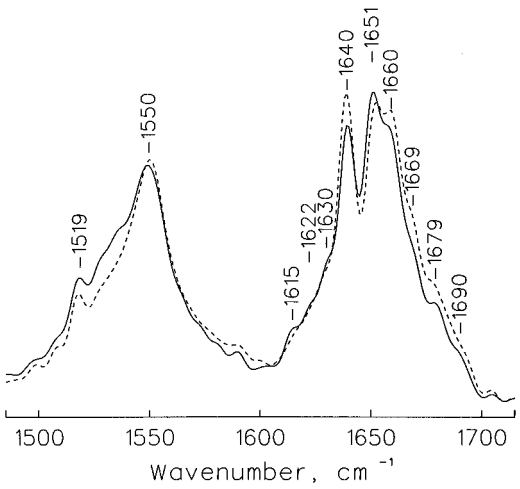

FIGURE 5: Deconvolved infrared spectra of UMP-kinase in H_2O buffer solution recorded at pH 7.4 (solid line) and at pH 9 (dashed line).

Table 2: Peak Positions and Relative Integrated Intensities of the Amide I Band Components of UMP-Kinase in H_2O Buffer[a]

pH 7		pH 9		pH 7 and 1 mM UTP	
position (cm^{-1})	area (%)	position (cm^{-1})	area (%)	position (cm^{-1})	area (%)
1691	4	1693	2	1694	2
1680	7	1679	13	1680	6
1669	11	1669	10	1670	12
1660	23	1661	18	1662	18
1651	21	1652	23	1652	27
1640	19	1639	21	1640	21
1622/30	15	1622/30	13	1622/30	14

[a] Assignments were as follows: 1622/1630 cm^{-1}, β-strands with strong hydrogen bonds (1622 cm^{-1} likely intermolecular hydrogen bonds); 1639/1640 cm^{-1}, β-strands with weaker intramolecular hydrogen bonds; 1650/1662 cm^{-1}, α-helix/irregular structures; 1669/1670 and 1680 cm^{-1}, turns; 1690/94 cm^{-1}, β-strands (antiparallel).

1620 and 1630 cm^{-1} are indicative of the presence of β-sheet structures with stronger hydrogen bonds, including intermolecular hydrogen bonds. The two major band components at 1651/1652 and 1660/1661 cm^{-1} represent overlapping absorptions assignable to α-helices and irregular polypeptide chains, while the band component at 1669 cm^{-1} can be attributed to turn structures. Turns and antiparallel β-sheet structures can also be correlated with band components at 1679/1680 and 1691 cm^{-1}. The absorption at 1615 cm^{-1} is indicative of tyrosine residues, which have a side-chain absorption at this frequency (Venyaminov & Kalnin, 1990).

Quantitative estimates of the percentages of the various secondary structures of UMP-kinase under different conditions were obtained by curve-fitting analysis of the amide I region and are given in Table 2. While these numbers should not be taken as absolute values due to the various assumptions inherent in curve-fitting (Jackson & Mantsch, 1995), they provide good estimates when used in a comparative-manner. Thus, increased pH (compare column 1 and 2 in Table 2) or binding of UTP to the enzyme (compare column 1 and 3 in Table 2) have only a minor impact on the secondary structure of UMP-kinase. The changes in the association behavior of UMP-kinase are obviously not related to any significant changes in the secondary structure of the protein.

Hydrodynamic Properties of UMP-Kinase. Gel permeation chromatography on Sephacryl S-300 HR of UMP-kinase in the presence of suitable molecular mass markers (rabbit muscle pyruvate kinase, lactate dehydrogenase and malate dehydrogenase) indicated that the molecular mass of the active enzyme is 150 ± 10 kDa, both at pH 6 and 9. The major soluble form of UMP-kinase (~70% of total protein) thus appears to be a hexamer (the molecular mass of the monomer is 25.8 kDa). The low molecular mass species (mono- or dimers) were catalytically inactive. The

Table 3: State of Oligomerization of UMP-Kinase from *E. coli* at Different pH Values As Determined from Sedimentation Velocity Experiments[a]

enzyme	pH	$S^0_{20,w}$	molecular mass	n-mer[b]	relative abundance (%)
wild-type	6.0	4.02	59 400	2.3 (2)	11
(0.1 mg/mL)		7.74	157 600	6.1 (6)	66
		16.32	482 900	18.7 (19)	23
wild-type	9.0	2.71	32 800	1.3 (1)	19
(0.37 mg/mL)		7.33	145 300	5.6 (6)	73
		11.8	297 400	11.5 (12)	8
D_{159}N mutant	7.4	2.85	35 300	1.4 (1)	<1
(0.6 mg/mL)		7.40	147 400	5.7 (6)	>99
D_{159}N mutant	9.0	2.68	32 100	1.2 (1)	17
(0.2 mg/mL)		7.36	146 400	5.7 (6)	83

[a] Experimental points representing the distribution of each molecular form were decomposed into Gaussian curves. The surface of one Gaussian is proportional to the concentration of the corresponding oligomeric form. Molecular masses were rounded up or down to the nearest hundred. [b] Numerical values for the n-mer of each species (number in parentheses) were obtained by dividing the molecular masses by 25 800 and rounding up or down to the nearest integer.

UMP-kinase species appearing in the void volume (~15% of total protein) exhibited a much lower specific activity than the hexamer.

From the equilibrium sedimentation experiments performed at three different speeds (data not shown), we found again a significant heterogeneity of UMP-kinase, both at pH 6 and 9. In addition to the hexameric forms, monomers, dimers, and high molecular mass oligomers were also observed. In 3 M GdmCl, single species of UMP-kinase corresponding to the unfolded monomer were identified. The distribution of various molecular forms of the bacterial enzyme was calculated from the velocity sedimentation experiments at pH 6 and 9. In the concentration range between 4 and 15 μM, preferential accumulation of a hexameric form of UMP-kinase was always observed, with no indication of a rapid equilibrium between monomers, dimers, or higher oligomers. A variant of UMP-kinase obtained by site-directed mutagenesis (D_{159}N) appeared at neutral pH almost exclusively as a hexamer (Table 3).

Analysis of D_{159}N Mutant of E. coli UMP-Kinase. While studying the effect of amino acid substitution on the catalytic and regulatory properties of *E. coli* UMP-kinase, we identified a variant (D_{159}N) whose solubility at neutral pH was considerably higher than that of the wild-type enzyme. Thus, after sonication in 50 mM Tris-HCl (pH 7.4) of bacteria overexpressing the D_{159}N mutant, the enzyme was recovered in the supernatant after centrifugation at 10000*g*. The purified D_{159}N mutant was stable at room temperature for several weeks. When this mutant was stored for several days at 4 °C at a concentration of 5 mg/mL, a slight precipitate was formed, but the specific activity of the soluble enzyme did not change. The kinetic constants for ATP and UMP of wild-type and of D_{159}N mutant determined at two pH values were similar (Table 4). Thus, at pH 6 both enzymes exhibited Michaelis−Menten kinetics with respect to substrates. At pH 7.4 (or 9, data not shown) both the wild-type and the mutant protein was inhibited by excess UMP. The K_m for UMP was 4−5 times lower at pH 7.4 than at pH 6.

Especially notable is the effect of pH on the sensitivity of UMP-kinase to allosteric effectors. While UTP inhibited the enzyme activity under all experimental conditions (Figure 6A−D), the strongest inhibition was observed at pH 7.4 and at low concentrations of UMP (Figure 6C). Since at pH 7.4 excess UMP inhibited the activity of UMP-kinase, these data suggest that, at high concentrations, the nucleoside monophosphate might compete with UTP for the allosteric site. The effect of GTP was also dependent on pH and on the concentration of UMP but independent of the concentration of ATP. Thus, at pH 7.4 and "high" (i.e., 1 mM) UMP concentration, GTP increased the activity of UMP-kinase by a factor of 3. At pH 6 or at "low" (i.e., 0.1 mM) UMP concentration, GTP exerted a dual effect, i.e., activation followed by inhibition as the nucleotide concentration was increased. These results suggest that GTP and UTP can bind to the same regulatory site on UMP-kinase but with different affinities and having different effects on the conformation of protein.

Table 4: Comparative Kinetic Properties of Wild-Type and of the D_{159}N Mutant of UMP-Kinase[a]

UMP-kinase	pH	K_m^{ATP} (mM)	K_m^{UMP} (mM)[b]	$V_m^{ATP,UMP}$ [μmol/(min/mg of protein)]
wild type	6.0	0.048	0.17	105
	7.4	0.120	0.043	128
D159N mutant	6.0	0.095	0.27	128
	7.4	0.286	0.052	153

[a] Experimental conditions are described in the legend of Figure 6. The apparent K_m for ATP and UMP was determined at a single fixed concentration of cosubstrates: 1 mM ATP and 0.1 mM (pH 7.4) or 1 mM (pH 6) UMP. The $V_m^{ATP,UMP}$ was obtained by extrapolating the reaction rates for infinite concentrations of ATP and UMP and by assuming that the concentration of one nucleotide substrate does not affect the apparent K_m of the second nucleotide substrate. [b] At pH 6.0, the reaction rates with UMP as variable substrate obeyed Michaelis−Menten kinetics. At pH 7.4, where inhibition by excess of UMP occurred, the reaction rates were fitted by the equation $v = V_m[UMP]/(K_m^{UMP} + [UMP] + [UMP]^2/K_I)$. The calculated K_I values were 1.2 mM for the wild-type enzyme and 0.65 mM for the D_{159}N mutant.

DISCUSSION

Protein solubility plays a fundamental role both *in vitro* and *in vivo*. In particular, the solubility properties control such diverse phenomena as aggregation of concentrated protein solutions *in vitro*, formation of inclusion bodies in genetically engineered organisms (Enfors, 1992), or pathogenesis of human diseases (Thomas et al., 1992; Welsh & Smith, 1993; Lorenzo & Yankner, 1994; Yu et al., 1995). The modulation of protein solubility by external factors (pH, temperature, salts, or dielectric constant) has been often used in separation of individual proteins (Green & Hughes, 1955). However, the mechanisms by which single amino acid substitutions can alter the solubility of proteins are yet poorly understood (Wetzel, 1994). The polymerization of sickle cell hemoglobin (HbS) is probably the best described protein self-assembly system (Eaton & Hofrichter, 1990). The β6 Glu → Val mutation in HbS affects a residue situated on the molecular surface and has little consequences on the functional properties of the protein in diluted solutions. However, at concentrations of hemoglobin found in the red cells, the Glu → Val substitution, which creates a "sticky" hydrophobic patch on the molecular surface, results in the polymerization of the tetramer upon deoxygenation (Eaton & Hofrichter, 1995). The formation of crystalline arrays of sickle cell hemoglobin is a classical example of how a ligand

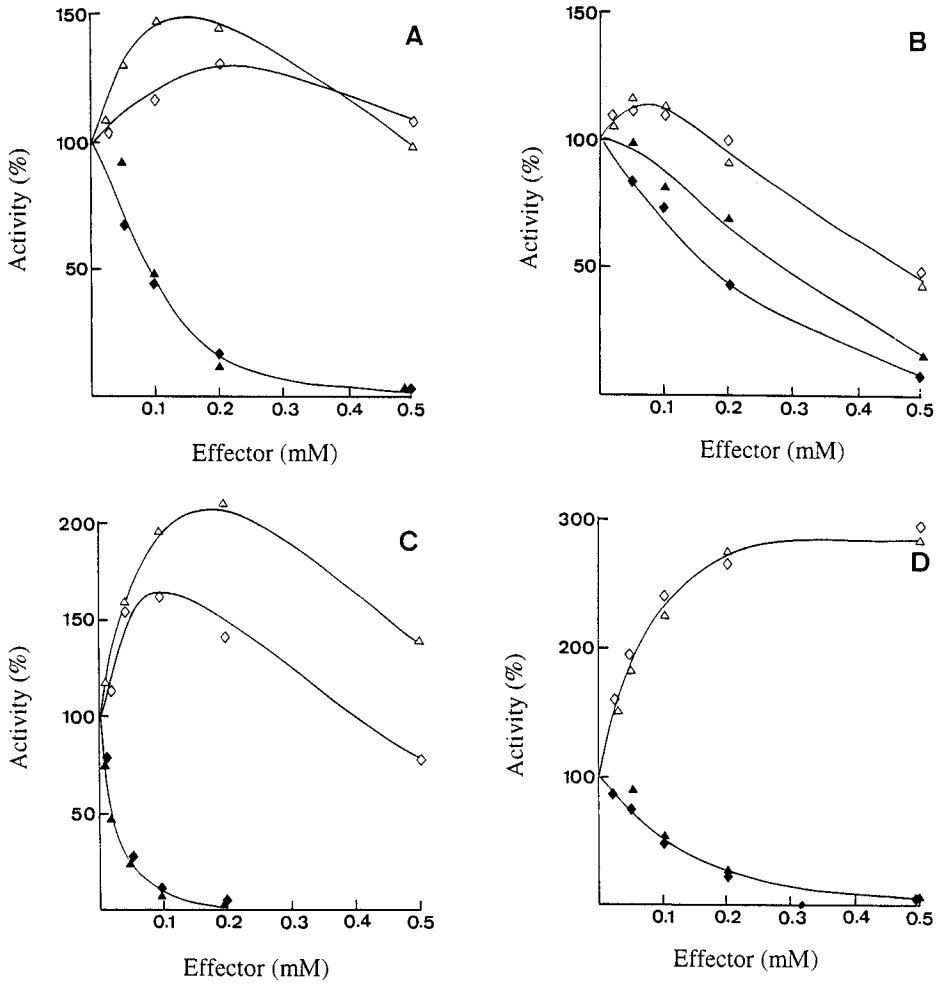

FIGURE 6: Dependence of the activity of wild-type and of the $D_{159}N$ mutant of UMP-kinase on UTP and GTP concentrations at two different pH values. The reaction medium buffered with 50 mM Tris-acetate at pH 6 (A and B) or with 50 mM Tris-HCl at pH 7.4 (C and D) contained 50 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 1 mM ATP, 0.1 mM (A and C), or 1 mM (B and D) UMP, various concentrations of UTP (▲, ◆) or GTP (△, ◇), and 2 units of each pyruvate kinase, NDP-kinase, and lactate dehydrogenase. The reaction was started with the wild-type (▲, △) or with the $D_{159}N$ mutant (◆, ◇) of UMP-kinase. 100% corresponds to the following activities: (A) 34 units/mg (wild-type) and 26 units/mg ($D_{159}N$); (B) 73 units/mg (wild-type) and 85 units/mg ($D_{159}N$); (C) 75 units/mg (wild-type) and 79 units/mg ($D_{159}N$); (D) 58 units/mg (wild-type) and 52 units/mg ($D_{159}N$).

(in this case, oxygen) can control the formation of a solid phase (Wyman & Gill, 1990).

The solubility properties of UMP-kinase from *E. coli* resemble in many respects those of hemoglobin. Thus, in the absence of a ligand (in this case, metal-free UTP), the protein exhibits low solubility at neutral pH. The observation that increased solubilization occurs only with uncomplexed UTP and that it is reversed by addition of Mg^{2+} indicates that a specific locus on the protein, which is not the catalytic site, is involved in the equilibrium between the soluble form and the aggregate. The effect of pH and of UTP on the solubility of UMP-kinase may be explained by the following putative mechanisms:

(i) A most trivial explanation would be that excess H^+ or OH^- increases the fraction of ionized species and thus shifts the equilibrium to the soluble form by favoring the repulsive forces between protein molecules. The pI of UMP-kinase, calculated from the amino acid composition, is 7.6. It is therefore understandable why the solubility of the bacterial enzyme is minimal at neutral pH and increases on either side of the isoelectric point.

(ii) Since the solubilizing effect of UTP is independent of pH, another explanation must also be considered. Aggregation of partially folded proteins is believed to be driven by hydrophobic interactions of side chains that are normally buried in the native state (Schein, 1990). We may thus postulate that the wild-type UMP-kinase hexamer, in the absence of UTP, adopts a conformation in which hydrophobic side chains are partially exposed. They can participate in intermolecular contacts which, at higher concentrations, leads to protein aggregation. Furthermore, we propose that conformational change of UMP-kinase upon binding of UTP decrease the exposure of hydrophobic surfaces. Indeed, this is supported by our preliminary experiments with a hydrophobic fluorescent probe ANS. Thus, UMP-kinase from *E. coli* considerably enhances the fluorescence of ANS. This effect, which depends on hydrophobic interactions between fluorophore and protein (Ewbank & Creighton, 1993), is decreased by μmolar concentrations of UTP. As expected, Mg^{2+} in excess restores the original surface hydrophobicity of UMP-kinase by removing UTP from its specific site (Figure 7).

(iii) By corroborating statement ii with the relative high solubility of the $D_{159}N$ mutant of the bacterial enzyme, we can assume that hydrophobic surfaces in the wild-type UMP-kinase are maintained by various interactions within each subunit, including ionic bonds between oppositely charged amino acids. Binding of UTP somehow disrupts salt bridges

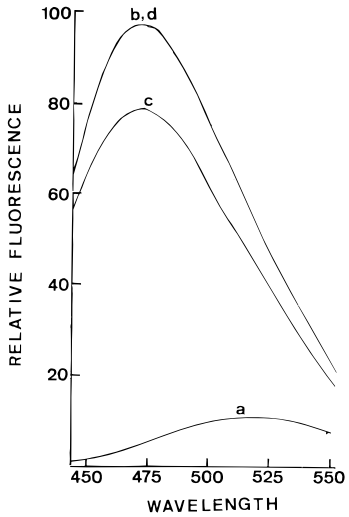

FIGURE 7: Fluorescence analysis of the interaction between UMP-kinase and ANS in the presence of UTP and Mg^{2+}. Emission spectra (λ_{exc} = 350 nm) of ANS (1 μM) in 50 mM Tris-HCl (pH 7.4) were recorded between 440 and 550 nm, (a) without additive, (b) in the presence of 2 μM UMP-kinase, (c) in the presence of 2 μM UMP-kinase and 10 μM UTP, and (d) in the presence of 2 μM UMP-kinase, 10 μM UTP, and 1 mM $MgCl_2$.

by a local or a propagated conformational effect rearranging the hydrophobic side chains. Moreover, if Asp_{159} is responsible for the low solubility of wild-type UMP-kinase by such a mechanism, it is conceivable that its positively charged counterpart would be a His residue. We can expect, therefore, that substitution of one of the three His residues (78, 96, or 211) with a polar uncharged amino acid residue would produce a soluble variant of UMP-kinase.

Whatever the cause of the low solubility of the *E. coli* UMP-kinase under *in vitro* conditions may be, a question arises: can the solubility (or lack thereof) of this enzyme play a role in the bacterial metabolism *in vivo*? Assuming that the UMP-kinase represents approximately 0.05% of all bacterial proteins (a number calculated on the basis of the specific activities of crude extracts and that of the pure enzyme), one finds that the cellular concentration of UMP-kinase is about 0.1 mg/mL, or 4 μM in terms of the monomer. Interestingly, this figure corresponds exactly to the solubility limit of UMP-kinase *in vitro*. From the total concentration of different NTPs in *E. coli* (Danchin et al., 1984), the concentration of UTP and that of Mg^{2+} (Snavely, 1990), we can assume that the concentration of Mg^{2+}-free UTP is between 2 and 3 μM. On the other hand, from kinetic and fluorescence experiments we found that the K_d of UTP/UMP-kinase complex is $\sim$1.5 μM. It results, therefore, that about 30−45% of all the cellular UMP-kinase is bound to metal-free UTP. Within the limits of the approximations used herein, it is conceivable that the physiological concentrations of divalent cations, or that of nucleotides in the cell, can affect not only the activity but also the state of aggregation of UMP-kinase.

The modulation by pH of the response of UMP-kinase to substrates and allosteric effectors is not that surprising. Subunit rearrangements into an oligomer, induced by changes in pH, were previously described for several other allosteric enzymes (Chen & Prusoff, 1978; Uyeda, 1979). Inhibition of activity by excess of UMP at pH 7.4 but not at pH 6 suggests that the nucleoside monophosphate binds to the allosteric UTP site most probably as deprotonated species, UMP^{2-}. This is in agreement with the fact that inhibition by UTP is higher at pH 7.4 than at pH 6 and is enhanced at low concentrations of UMP.

In conclusion, UMP-kinase from *E. coli* is not only a new allosteric enzyme involved in the synthesis of pyrimidine nucleotides, but, due to its particular physicochemical properties, it is also a very interesting model for exploring structural factors involved in refolding and solubilization of proteins under normal or pathological conditions.

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REFERENCES

Anderson, E. P. (1973) Nucleoside and Nucleotide Kinases, in *The Enzymes* 3rd ed., (Boyer, P. D., Ed.) Vol. 8, pp 49−96, Academic Press, New York and London.

Bârzu, O., Kiss, L., Bojan, O., Niac, G., & Mantsch, H. H. (1976) *Biochem. Biophys. Res. Commun. 73*, 894−902.

Blondin, C., Serina, L., Wiesmüller, L., Gilles, A.-M., & Bârzu, O. (1994) *Anal. Biochem. 220*, 219−221.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Chen, M. S., & Prusoff, W. H. (1978) *Methods Enzymol. 51*, 354−360.

Cohn E. J., & Edsall, J. T. (1943) in *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, p 157, Reinhold, New York.

Danchin, A., Dondon, L., & Daniel, J. (1984) *Mol. Gen. Genet. 193*, 473−478.

Eaton, W. A., & Hofrichter, J. (1990) *Adv. Protein Chem. 40*, 63−279.

Eaton, W. A., & Hofrichter, J. (1995) *Science 268*, 1142−1143.

Enfors, S. O. (1992) *Trends Biotechnol. 10*, 310−315.

Ewbank, J. J., & Creighton, T. E. (1993) *Biochemistry 32*, 3694−3707.

Freire, E., van Osdol, W. W., Mayorga, O. L., & Sanchez-Ruiz, J. M. (1990) *Annu. Rev. Biophys. Biophys. Chem. 19*, 159−188.

Green, A. A., & Hughes, W. L. (1955) *Methods Enzymol. 1*, 67−90.

Jackson, M., & Mantsch, H. H. (1995) *Crit. Rev. Biochem. Mol. Biol. 30*, 95−120.

Konrad, M. (1992) *J. Biol. Chem. 267*, 25652−25655.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Liljelund, P., Sanni, A., Friesen, J. D., & Lacroute, F. (1989) *Biochem. Biophys. Res. Commun. 165*, 464−473.

Lorenzo, A., & Yankner, B. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12243−12247.

Mantsch, H. H., Moffattt, D. J., & Casal, H. L. (1988) *J. Mol. Struct. 173*, 285−298.

Müller-Dieckmann, H.-J., & Schulz, G. E. (1994) *J. Mol. Biol. 236*, 361−367.

Neuhard, J., & Nygaard, P. (1987) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., & Umbarger, H. E., Eds) pp 445−473, American Society for Microbiology, Washington, D.C.

Philo, J. S. (1994) in *Modern Analytical Ultracentrifugation: Acquisition and Interpretation of Data for Biological and Synthetic Polymer System* (Schuster, T. M., & Laue, T. M., Eds) pp 156−170, Birkhäuser, Boston.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanchez-Ruiz, J. M., Lopez-Lacomba, J. L., Cortijo, M., & Mateo, P. L. (1988) *Biochemistry 27*, 1648−1652.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Schein, C. H. (1990) *Biotechnology 8*, 308−317.
Serina, L., Blondin, C., Krin, E., Sismeiro, O., Danchin, A., Sakamoto, H., Gilles, A.-M., & Bârzu, O. (1995) *Biochemistry 34*, 5066−5074.
Smallshaw, J. D., & Kelln, R. A. (1992) *Life Sci. Adv. 11*, 59−65.
Snavely, M. D. (1990) in *Metal Ions in Biological Systems* (Sigel, H., & Sigel, A., Eds) Vol. 26, pp 155−175, Marcel Dekker Inc., New York.
Surewicz, W. K., Mantsch, H. H., & Chapman, D. (1993) *Biochemistry 32*, 389−394.
Thomas, P. J., Ko, Y. H., & Pedersen, P. L. (1992) *FEBS Lett. 312*, 7−9.
Uyeda, K. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 193−244.
Venyaminov, S. Yu, & Kalnin, N. N. (1990) *Biopolymers 30*, 1243−1257.
Welsh, M. J., & Smith, A. E. (1993) *Cell 73*, 1251−1254.
Wetzel, R. (1994) *Trends Biotechnol. 12*, 193−198.
Wiesmüller, L., Noegel, A. A., Bârzu, O., Gerisch, G., & Schleicher, M. (1990) *J. Biol. Chem. 265*, 6339−6345.
Wyman, J., & Gill, S. J. (1990) in *Binding and Linkage. Functional Chemistry of Biological Macromolecules*, p 237, University Science Books, Mill Valley, CA.
Yamanaka, K., Ogura, T., Niki, H., & Hiraga, S. (1992) *J. Bacteriol. 174*, 7517−7526.
Yang, J. T., Wu, C. S., & Martinez, H. M. (1986) *Methods Enzymol. 130*, 208−269.
Yu, M.-H., Lee, K. N., & Kim, J. (1995) *Nature Struct. Biol. 2*, 363−367.

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