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Specific Derivatization of the Active Site Tyrosine in dUTPase Perturbs Ligand Binding to the Active Site

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Selective modification of one (of three) tyrosine residue per enzyme monomer leads to inactivation of dUTPase of the retrovirus equine infectious anemia virus (EIAV). The substrate dUMP and the cofactor Mg^{2+} protects against inactivation and modification, in agreement with the study on *E. coli* dUTPase (Vertessy *et al.* (1994) *Biochim. Biophys. Acta* **1205,** 146–150). Amino acid analyses of nitrated dUTPases confirmed Tyr-selectivity of modification. The nitrated residue in *E. coli* dUTPase was identified as the evolutionary highly conserved Tyr-93. The modifiable residue is shown to be the only Tyr exposed in both *E. coli* and EIAV dUTPases. As a consequence of Tyr-93 derivatization, the Mg^{2+}-dependent interaction between the substrate-analogue dUDP and *E. coli* dUTPase becomes impaired as shown by circular dichroism spectroscopy, here presented as a tool for monitoring ligand binding to the active site.

The ubiquitous enzyme dUTP nucleotidohydrolase (dUTPase) catalyses the pyrophosphorolysis of dUTP. dUTPase provides dUMP, the substrate for thymidylate synthase, and maintains low dUTP/dTTP ratios, protecting against uracil incorporation into DNA (1). Extensive excision repair of uracil-substituted DNA leads to DNA fragmentation and cell death (2). Null mutations in the dUTPase gene are lethal in such diverse organisms as *E. coli* (3) and yeast (4) indicating a generally essential role for the enzyme.

dUTPase might provide new insights into antiviral and anticancer chemotherapy. Mutations in viral dUTPase genes strongly impair virulence of herpes simplex virus type 1 (5) and the retrovirus EIAV (6) in non-proliferating cells. Resistance to the widely used antineoplastic drug fluorodeoxyuridine correlates to an elevation of dUTPase activity (7), raising the hope that dUTPase inhibitors may improve therapeutic effectiveness. In fact, membrane-permeable substrate-analogous inhibitors of dUTPase were demonstrated to be efficient against human cancer cells *in vitro* (8).

Protein sequence data for the enzyme from diverse organisms indicate five conserved motifs (Motifs 1 to 5) (9). Crystal structure of the *E. coli* enzyme reveals a trimeric arrangement of identical subunits (10). Unfortunately, the active site could not be identified with certainty. Recently, we showed that one of the two tyrosine residues of *E. coli* dUTPase is probably involved in the active site (11). In the present work we identify this residue as the conserved Tyr-93 of Motif 3 in *E. coli* dUTPase, assess the importance of tyrosine residue(s) for the function of EIAV dUTPase, characterize the exposed/buried tyrosine residues in the enzyme from both sources and demonstrate an impaired binding of the substrate analogue dUDP to the modified enzyme using circular dichroism difference spectroscopy.

MATERIALS AND METHODS

Enzyme preparation and assay. dUTPase from EIAV and *E. coli* was purified as previously (12, 13). Protein concen-

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Abbreviations: dUTPase, deoxyuridine triphosphate nucleotidohydrolase, EC 3.6.1.23; EIAV, equine infectious anemia virus; $K_{d,app}$, apparent dissociation constant.

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tration was measured spectrophotometrically using $A^{0.1\%}$ 1 cm, 280 $^{=0.52}$ for *E. coli* dUTPase (determined in this study by using amino acid analysis data to quantify protein concentration), a value in good agreement with that predicted from the amino acid composition (c.f. (13)) and $A^{0.1\%}$ 1 cm, $280^{=1.05}$ for EIAV dUTPase (12). Molecular mass of the trimeric *E. coli* enzyme is 48 kDa (10), that of the trimeric EIAV enzyme is 41 kDa (12). Throughout the present study, enzyme concentrations refer to the monomeric species. Before use, aliquots of the enzyme were dialyzed against respective buffers. Enzyme activity was assayed at enzyme concentrations 25–50 nM in the presence of 40 μM dUTP (Sigma) and 100 μM Mg^{2+} in 1 mM Tes/HCl, pH 7.5 containing 0.15 M KCl and 40 μM Phenol Red indicator (Merck) (assay buffer). Proton release during the transformation of dUTP into dUMP and PPi was followed at 559 nm at 25°C (14, 15). Alternatively, activity at 25°C was measured with a chromatographic assay (16). Both methods gave the same specific activity of 23000 nmol dUTP/mg dUTPase/min for *E. coli* and 10500 nmol dUTP/mg dUTPase/min for EIAV dUTPase, in good agreement with previous data (12, 13).

Chemical derivatization with tetranitromethane (Aldrich) or N-acetylimidazole (Sigma) and spectral determination of modified tyrosine residues was performed as previously (11) following the methods of Riordan and Vallee (17), at room temperature. Briefly, an aliquot from a fresh dilution of tetranitromethane in 95% (v/v) ethanol was added to 60–120 μM dUTPase in 0.05 M Tris/HCl, pH 8.9, containing 0.15 M KCl with or without 5 mM Mg^{2+} and dUMP. Molar excess of tetranitromethane over dUTPase was 2.5-10-fold. Alternatively, solid N-acetylimidazole in a 260-fold molar excess over dUTPase was added to 90–120 μM enzyme in 0.01 M Tris/HCl, pH 7.5, containing 0.15 M KCl with or without 5 mM Mg^{2+} and dUMP. Deacetylation was performed by the addition of 0.77 M hydroxylamine to the acetylated enzyme. After modification, reagents were removed by dialysis.

UV and VIS spectral titration of native and nitrated enzymes. Enzyme, 30–60 μM in 10 mM Bistris-Propane- 10 mM Glycine- 150 mM KCl buffer, was titrated at 25°C by sequential addition of 0.3-1 μL of 1 M HCl or 2 M KOH in a cuvette. After each addition, pH was measured by means of a microelectrode and spectra were recorded in the range of 200–650 nm. Reversibility of the titration was checked by back titration after reaching pH 11.0, 11.5 and 12.0. Spectrophotometric measurements in thermostatted cuvettes were carried out on a JASCO-V550 UV/VIS spectrophotometer equipped with a data processing system.

Circular dichroism measurements. Near-UV circular dichroism spectra (230–350 nm) were recorded on a JASCO 720 spectropolarimeter using 10-mm cuvettes thermostatted at 25°C. Native and modified dUTPase from *E. coli* at a concentration of 45 μM was titrated by addition of dUDP (0-100 μM) in 10 mM sodium-phosphate - 150 mM KCl buffer with or without 1 mM $MgCl_2$, pH 7.0. Three scans of every spectrum were averaged. Spectra measured immediately after mixing enzyme and dUDP were stable for at least 30 min.

Amino acid analysis was carried out on native and nitrated dUTPase at the University of Uppsala. Samples were hydrolyzed in 6 M HCl at 110°C for 24 h.

Edman degradation of nitrated *E. coli* dUTPase was carried out at the Department of Biochemistry, University of Saarland, using the Protein Sequencing System model 473A from Applied Biosystems. The phenylthiohydantoin derivative of authentic 3-nitrotyrosine elutes after diphenylthiourea, before the tryptophan peak (18) well separated from the peak of the derivative of unmodified tyrosine.

RESULTS AND DISCUSSION

Chemical Modification of dUTPase and Analysis of Products

Incubation of EIAV dUTPase with N-acetyl imidazole or tetranitromethane for 30 min resulted in (80+/−5)% activity loss. Addition of 0.77 M NH_2OH to the acetyl dUTPase led to deacetylation in 15 min, as followed by absorbance change at 278 nm (cf. (17)), and activity was completely restored. The presence of the substrate dUMP and the cofactor Mg^{2+} significantly decreased the extent of inactivation and modification (Table 1). Amino acid analyses of nitrated EIAV and *E. coli* dUTPase samples proved an exclusive selectivity of nitration towards tyrosine (Table 2). Nitrotyrosine content determined from the amino acid analysis correlates well with the results of the spectrophotometric determination, i.e. only about one tyrosine residue/enzyme monomer is modified under our experimental conditions in either *E. coli* or EIAV dUTPase. These results, in parallel with our previous experiments with the *E. coli* dUTPase (11), suggest the active site involvement of a tyrosine residue in the EIAV enzyme, as well.

Solvent Accessibility of Tyrosine Residues in E. coli and EIAV dUTPases

The increased susceptibility of one single Tyr out of two (*E. coli*) or three (EIAV) per monomeric enzyme may be accounted for by assuming differences in solvent accessibility. Increase in absorbance of the tyrosinate anion as compared to protonated tyrosine at the characteristic wave-

TABLE 1
Protection of EIAV dUTPase against Modification in the Presence of Effectors

Modification reagent	Effectors	Activity in % of control	Modified Tyr residues/ monomeric dUTPase
N-acetylimidazole	−	21	0.7
N-acetylimidazole	+	45	0.4
Tetranitromethane	−	15	0.8
Tetranitromethane	+	80	0.2

80 μM EIAV dUTPase was incubated with 21 mM N-acetylimidazole or 640 μM tetranitromethane for 30 min. Activity and modified Tyr content were assayed spectrophotometrically (see Methods). Data represent mean of three experiments, error of activity determinations was 5%, that of modified Tyr determinations was 15%. Effectors (5 mM dUMP and 5 mM Mg^{2+}) were presented as indicated.

length 295 nm can be used to quantitatively follow tyrosine titration in proteins (18). Spectroscopic titration of native dUTPases from both sources resulted in two-step proton-dissociation curves (Fig. 1. closed symbols). pK_a values for the *E. coli* enzyme would be 10.25 and 11.8, one residue titrated in each step; pK_a values in the EIAV dUTPase were found to be 10.0 and 12.0, with one residue titrated in the first and two in the second step. The titration could not be reversibly followed up to total deprotonation of the residues in the second step possibly due to protein denaturation. Limit of reversibility was encountered at pH 11.5 in both enzymes, points of back titration after reaching pH

TABLE 2
Amino Acid Composition of Native and Nitrated *E. coli* and EIAV dUTPases

	E. coli			EIAV		
Residue	Native sample	Predicted	Nitrated sample	Native sample	Predicted	Nitrated sample
Asn + Asp	16.1	16	16.5	15.0	15	15.8
Thr	7.2	7	7.2	7.1	7	6.7
Ser	8.0	8	8.2	9.5	9	9.1
Glu + Gln	13.0	13	13.2	15.9	16	16.6
Pro	8.7	9	8.8	6.4	6	6.4
Gly	17.6	18	17.8	15.4	15	15.7
Ala	12.5	12	12.4	4.8	4	4.9
Half-cystine	0.8	1	0.9	1.9	2	1.8
Val	11.3	11	11.4	7.6	7	7.5
Met	6.0	6	5.7	2.2	3	2.3
Ile	9.5	10	9.5	16.1	17	16.4
Leu	15.7	16	16.8	7.2	7	7.4
Tyr	2.3	2	1.1	3.0	3	1.9
Phe	6.0	6	5.7	4.4	5	4.2
His	3.8	4	3.8	1.7	2	1.6
Lys	5.4	5	5.0	10.5	11	10.0
Arg	6.9	7	6.9	3.2	3	3.2
Nitro-Tyr	—	—	0.8	—	—	0.7

Analysis was performed on samples hydrolyzed in 6 M HCl, 24 h, 110°C and results are presented as number of residues per polypeptide. Tryptophan was not determined. The composition predicted from the protein sequence (*E. coli*) or DNA sequence (EIAV) is included for comparison.

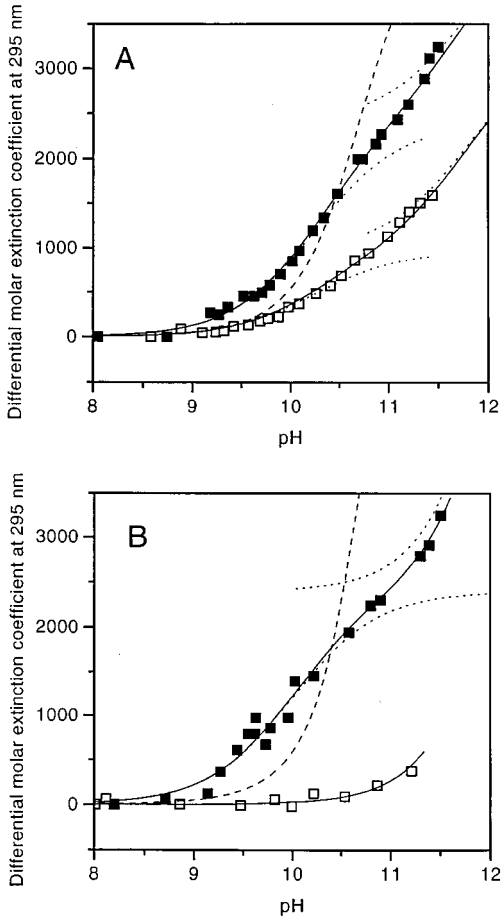

FIG. 1. UV spectral titration of native (■) and nitrated (□) *E. coli* (A) and EIAV (B) dUTPase. NO_2-Tyr content/monomeric enzyme was 0.6 mole for *E. coli* and 0.9 mole for EIAV dUTPase. Titration of one phenolic group caused a change of 2400 in molar extinction coefficient measured at 295 nm, in good agreement with (18). **Solid lines** are proton dissociation curves, calculated as given below and fitted to the data points. For native *E. coli* dUTPase, the equilibria are $H_2A = H^+ + HA^-$ and $HA^- = A^{2-} + H^+$ with dissociation constants $pK_{a1} = 10.25$ and $pK_{a2} = 11.8$, respectively. Protein concentration $c = [H_2A] + [HA^-] + [A^{2-}]$ and the differential molar extinction coefficient $\Delta\epsilon = (2400[HA^-]+4800[A^{2-}])/c$. For nitrated *E. coli* dUTPase, $\Delta\epsilon = (0.4 \times 2400[HA^-] + 3360[A^{2-}])/c$, since 0.6 mol Tyr/protein is nitrated and titrates at $pK_a = 7.6$ (11). For EIAV dUTPase, three equilibria hold: $H_3A = H^+ + H_2A^-$; $H_2A^- = HA^{2-} + H^+$ and $HA^{2-} = A^{3-} + H^+$ with $pK_{a1} = 10$ and $pK_{a2} = pK_{a3} = 12.0$. Here, $c = [H_3A] + [H_2A^-] + [A^{3-}]$ and $\Delta\epsilon = (2400[H_2A^-] + 4800[HA^{2-}] + 7200[A^{3-}])/c$. For the nitrated EIAV protein $\Delta\epsilon = (2400[HA^{2-}] + 4800[A^{3-}])/c$, since approx. one (0.9) mole Tyr/protein is nitrated and titrates at $pK_a = 7.6$ (not shown). **Dashed lines** were calculated assuming one class of tyrosine residues in the native proteins with the same $pK_a = 10.7$ for every group. **Dotted lines** fit the different classes of titrated groups separately, where applicable.

11.5 fell on the forward titration curve, and spectra were stable for at least 75 min at pH < 11.5. Consequently, the pK_a assigned to the residues titrating in the higher pH range should only be regarded as an approximation. Nevertheless, results prove the existence of two different classes of tyrosines in both enzymes. Assuming the same pK_a for all phenolic hydroxyl groups, calculated curves clearly fail to describe the data points (c.f. dashed lines in Fig. 1. are too steep for any pK_a to fit the data). As a result of nitration, the first portion on the dissociation curve was diminished (partially nitrated *E. coli* dUTPase, Fig. 1.A, open symbols) or erased (nitrated EIAV dUTPase with 0.9 residues of 3-nitro Tyr per monomer, Fig. 1.B, open symbols), suggesting that modification

selectively involves the residue with pK_a around 10. We conclude that for both dUTPases, the Tyr modifiable is the only solvent accessible Tyr in the enzyme, while other tyrosine residues (one in *E. coli* and two in EIAV) behave as non-accessible.

Identification of the Modifiable Tyr

Edman degradation of nitrated *E. coli* dUTPase was carried out up to the 24th residue. The sequence determined was: MMKKIDVKILDPRVGKEFPLPTYA, confirming previous results (10). The phenylthiohydantoin derivative of tyrosine in the 23rd cycle eluted in a single peak at the position of the unmodified tyrosine (not shown). No distinct peak could be resolved between the retention times of diphenylthiourea and tryptophan, where the derivative of 3-nitrotyrosine elutes (19). This result provides strong support for the selective modification of Tyr-93 during nitration, since there are only two tyrosine residues in the *E. coli* enzyme. Tyr-93 is a strongly conserved residue within the conserved Motif 3 of dUTPases and is also present in the EIAV enzyme. In our search of Swiss-Prot and GenBank databases, it is conservatively replaced by Phe in 4 cases out of the total 30 investigated. Functional importance of Tyr-93 in other species is reflected by insertional mutagenesis: its replacement by a five-residue insert in the enzyme from feline immunodeficiency virus (20) or by a four-residue insert in the enzyme from herpes simplex virus (21) renders the enzymes inactive. In the crystal structure of the *E. coli* dUTPase, Tyr-93 shows up as accessible to solvent, while Tyr-23 is located in a hydrophobic environment (10). In the previous section we showed that the modifiable Tyr is the only solvent-exposed one. These two observations together provide independent support for Tyr-93 as the target of modification and permit localization of the active site of the bacterial enzyme in the shallow depression seen in the crystal structure (10), formation of which involves other conserved residues as well. It seems likely that the Tyr in the EIAV enzyme, which we observe as exposed to solvent and modification, is also the conserved Tyr of Motif 3 as in the *E. coli* enzyme.

Interaction of the Substrate Analogue dUDP with Native and Modified dUTPase

To characterize the effect of modification on ligand binding, a sensitive spectroscopic technique was sought. The presence of the substrate analogue dUDP exerted no major effect on the intrinsic protein flourescence of *E. coli* dUTPase (not shown). On the other hand, near-UV circular dichroism spectra were significantly perturbed in the presence of dUDP. The near UV circular dichroism spectrum of native *E. coli* dUTPase shows several fine structured bands in the 250–300 nm wavelength range, while the spectrum of dUDP is characterized by one peak with maximum at 270 nm (Fig. 2.A). Ellipticity at 270 nm was strictly linearly proportional to dUDP concentration up to 100 μM, if ligand was present alone (not shown). An equimolar mixture of dUDP and dUTPase exhibits a significant difference circular dichroism spectrum as compared to the spectra of the components measured alone (Fig. 2.A). Most characteristically, ellipticity at 270 nm is enhanced in the spectrum of the mixture in a dUDP-concentration dependent, saturable manner (Fig. 2.B, closed squares). Our interpretation of this phenomenon states that complex formation of enzyme and dUDP is reflected in an increase of ellipticity at 270 nm. Data were fitted by using $K_{d,app.} =$ 10 μM as the dissociation constant of the one to one enzyme-dUDP complex. The dUTPase-dUDP interaction is found to be Mg^{2+}-dependent, since titration in the absence of added Mg^{2+} significantly diminishes the spectropolarimetric signal (Fig. 2.B, closed circles, data fitted with $K_{d,app} =$ 170 μM).

Similar experiments conducted with derivatized dUTPase in the presence of Mg^{2+} revealed that enhancement of ellipticity at 270 nm in the modified enzyme-ligand mixture is dramatically diminished as compared to the signal displayed by the native enzyme-ligand mixture (Fig. 2.B, open symbols). These results suggest that Tyr-modification perturbs the Mg^{2+}-dependent dUDP binding site on the enzyme.

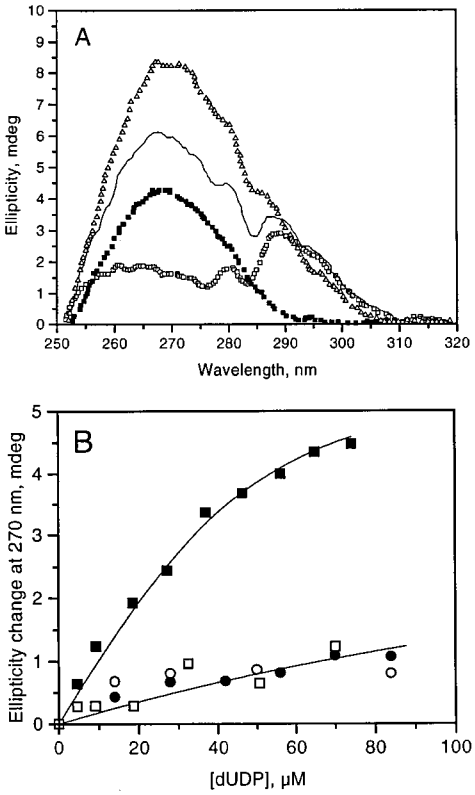

FIG. 2. (A) Near-UV circular dichroism spectra of *E. coli* dUTPase (45 μM. □), dUDP (46 μM. ■), and the mixture thereof (△). Solid line shows the calculated sum of the spectra of enzyme and ligand, measured separately. (B) Titration of *E. coli* dUTPase with dUDP (native enzyme: with (■) or without (○) 1 mM $MgCl_2$; modified enzyme with 1 mM $MgCl_2$: nitrated (□) or acetylated (●). At each dUDP concentration, ellipticity change ($\Delta\Theta$) at 270 nm was calculated by subtracting the ellipticity of dUDP and that of the enzyme, measured separately, from the ellipticity measured in the mixture. Data gained with native dUTPase +/− Mg^{2+} were fitted to the equation: $\Delta\Theta = (\Delta[\Theta]/2)((a-(a\text{^}2 - 4c_1c_2)\text{^}0.5)$, where $\Delta[\Theta]$ is the differential molar ellipticity of the dUDP–dUTPase complex, $a = c_1 + c_2 + K_{d,app}$, c_1 and c_2 are enzyme and dUDP concentrations, respectively (solid lines). $\Delta[\Theta]$ was 0.091 mdeg/μM for both data sets, $K_{d,app}$ was 10 μM or 170 μM, in the presence or absence of Mg^{2+}, respectively.

We conclude that the evolutionarily conserved Tyr of Motif 3 is an exposed active-site residue implicated in the Mg^{2+}-dependent binding of dUDP in *E. coli* dUTPase. Most recent X-ray data on the dUTPase-dUDP complex in the absence of Mg^{2+} also locate Tyr-93 in the dUDP-binding pocket (22). A single exposed tyrosine residue, probably corresponding to the conserved one, was presently found to be involved in the function of the EIAV enzyme, as well, suggesting a general functional importance of this residue. With an intention of detailed structural characterization, nitro- and acetyl-derivatives of *E. coli* dUTPase are being crystallized in our laboratory for future X-ray studies. Further mutagenetic and structural studies exploring Motif 3 and other conserved residues will hopefully aid inhibitor design.

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REFERENCES

1. Kornberg, A., and Barker, T. A. (1991) DNA Replication, 2nd ed., Freeman, New York.
2. Ingraham, H. A., Dickey, L., and Goulian, M. (1986) *Biochemistry* **25,** 3235–3230.
3. El-Hajj, H. H., Zhang, H., and Weiss, B. (1988) *J. Bacteriol.* **170,** 1069–1075.
4. Gadsden, M. H., McIntosh, E. M., Game, J. C., Wilson, P. J., and Haynes, R. H. (1993) *EMBO J.* **12,** 4425–4431.
5. Pyles, R. B., Sawtell, N. M., and Thompson, R. L. (1992) *J. Virol.* **66,** 6706–6713.
6. Threadgill, D. S., Steagall, W. K., Flaherty, M. T., Fuller, F. J., Perry, S. T., Rushlow, K. E., LeGrice, S. F. J., and Payne, S. L. (1993) *J. Virol.* **67,** 2592–2600.
7. Canman, C. E., Radany, E. H., Parsels, L. A., Davis, M. A., Lawrence, T. S., and Maybaum, J. (1994) *Cancer Research* **54,** 2296–2298.
8. Zalud, P., Wachs, W. O., Nyman, P. O., and Zeppezauer, M. (1995) *in* Purine and Pyrimidine Metabolism in Man VIII (Sahota, A., and Taylor, M., Eds.), pp. S.135–138, Plenum, New York.
9. McGeoch, D. J. (1990) *Nucleic Acids Res.* **18,** 4105–4110.
10. Cedergren-Zeppezauer, E. S., Larsson, G., Nyman, P. O., Dauter, Z., and Wilson, K. S. (1992) *Nature (London)* **355,** 740–742.
11. Vertessy, B. G., Zalud, P., Nyman, P. O., and Zeppezauer, M. (1994) *Biochim. Biophys. Acta*, **1205,** 146–150.
12. Bergman, A.-C., Bjornberg, O., Nord, J., Rosengren, A. M., and Nyman, P. O. (1995) *Protein Expression and Purification*, **6,** 379–387.
13. Hoffmann, I., Widström, J., Zeppezauer, M., and Nyman, P. O. (1987) *Eur. J. Biochem.* **164,** 45–51.
14. Clark, D. D., Daggett, S. G., and Schuster, S. M. (1984) *Arch. Biochem. Biophys.* **233,** 378–392.
15. Zalud, E. (1989) Diplom Thesis, Univ. Saarbrücken, Germany.
16. Hoffmann, I. (1988) Ph. D. Thesis, Univ. Saarbrücken, Germany.
17. Riordan, J. F., and Vallee, B. L. (1972) *Methods Enzymol.* XXV (Hiss, C. H. W., and Timasheff, S. N., Eds.), pp. 506–521, Acad. Press, New York.
18. Tanford, C., Hauenstein, J. D., and Rands, D. G. (1956) *J. Am. Chem. Soc.* **77,** 6409–6413.
19. Shimokawa, T., Kulmacz, R. J., DeWitt, D. L., and Smith, W. L. (1990) *J. Biol. Chem.* **265,** 20073–20076.
20. Wagaman, P. C., Hasselkus-Light, C. S., Henson, M., Lerner, D. L., Phillips, T. R., and Elder, J. H. (1993) *Virology* **196,** 451–457.
21. Fisher, F. B., and Preston, V. G. (1986) *Virology* **148,** 190–197.
22. Larsson, G. (1995) Ph.D. Thesis, University of Lund, Sweden.