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Identification of reactive tyrosine residues in cysteine-reactive dehydrogenases

Differences between liver sorbitol, liver alcohol and *Drosophila* alcohol dehydrogenases

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Modification of tyrosine residues with tetranitromethane and reversible sulphite protection of cysteine residues were tested on three dehydrogenases of two families. In liver alcohol dehydrogenase no Tyr residue is appreciably labelled, while in the homologous sorbitol dehydrogenase Tyr-109 is specifically labelled; the difference corresponds to a segment correlating with subunit interactions and the different quaternary structures of the proteins. In *Drosophila* alcohol dehydrogenase, Tyr modification is multiple, and the results show the presence of two different states of Cys residues, reactive in the presence and absence of cupric ions, respectively. Super-activation with cyanide was also noticed after *S*-sulphocysteine protection. The results demonstrate the possibility of identification of specific Tyr residues in proteins with reversibly protected Cys residues.

S-Sulphocysteine; Tetranitromethane; Reversible cysteine modification; Nitrotyrosine; Enzyme inactivation; Reactive residue

1. INTRODUCTION

Chemical modifications with side chain-specific reagents offer modes of identifying residues at active sites of proteins. In the case of tyrosine residues, tetranitromethane is a fairly specific reagent, but cysteine residues can also react and reduce the usefulness of the reagent [1]. Dehydrogenases illustrate the problem well, often having both important cysteine and tyrosine residues. In short-chain dehydrogenases (including *Drosophila* alcohol dehydrogenase [2,3]), a reactive tyrosine residue has been postulated [4], but labelled only in 3α/20β-hydroxysteroid dehydrogenase [5], apparently lacking cysteine residues. In medium-chain dehydrogenases of the liver alcohol dehydrogenase type, reactive Cys residues [6–11] constitute ligands to metal atoms [12,13] and complicate identification of reactive tyrosine residues (cf. [5]). Use of reversible Cys modification would be of interest, to get products that, after deblocking of Cys-protective groups, are only Tyr-modified.

For that reason, we have studied reversible cysteine modification, coupled with Tyr modification, utilizing two cysteine-rich dehydrogenases, the liver alcohol and sorbitol dehydrogenases, and one short-chain dehydrogenase, *Drosophila* alcohol dehydrogenase. We find the old method of sulphite modification of cysteine residues in the presence of Cu^{2+}, to give the *S*-sulphocysteine derivative [14–16], useful as a mild means of protection. In the absence of intervening tetranitromethane treatment, this procedure is reversible, also for the cysteine-rich and metal-containing mammalian enzymes, and in the presence of tetranitromethane reactive tyrosine residues can be differentiated. Results correlate with enzyme inactivation and conformational properties. In addition, unexpected relationships are found regarding the two cysteine residues of the *Drosophila* enzyme, where sulphite reaction in the absence of Cu^{2+}, and super-reactivation with cyanide, may correlate with native oxidation or adduct formation.

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2. MATERIALS AND METHODS

2.1. *Dehydrogenases and enzyme assays*

Alcohol dehydrogenase from horse liver and sorbitol dehydrogenase from sheep liver were obtained from Sigma (MO), *Drosophila* alcohol dehydrogenase was prepared as described [17]. Enzyme activities were measured at 340 nm, 25°C, using oxidation of NADH with D-fructose for sorbitol dehydrogenase at pH 7.4, reduction of NAD with ethanol for liver alcohol dehydrogenase at pH 10, and with 2-propanol for *Drosophila* alcohol dehydrogenase at pH 8.6.

2.2. *Chemical modifications*

Enzyme samples in 0.1 M sodium phosphate, pH 8.0, were sulphated with 5 mM sodium sulphite in the presence of 0.05 mM $CuSO_4$ at room temperature in the phosphate buffer. Tyrosine residues were nitrated with tetranitromethane at room temperature with

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a 1- to 40-fold molar excess of reagent in the same buffer. Reduction of sulphated, or sulphated and nitrated enzymes, was performed with 5 mM dithiothreitol at room temperature for 1 h in sodium phosphate buffer. Excess reagents at each step of enzyme modification were removed by dialysis against phosphate buffer.

2.3. *Structural analysis*

Enzymes modified with tetranitromethane were dialyzed against water, lyophilized, carboxymethylated, analyzed for nitrotyrosine content by amino acid analysis after hydrolysis, and submitted to peptide analysis for identification of the labelled tyrosine residue(s) after cleavage with Lys-C protease, and peptide purifications by reverse-phase high performance liquid chromatography [4]. For cleavage, samples were solubilized in 9 M urea, diluted to 1.5 M urea with 0.1 M ammonium bicarbonate, and treated with proteinase Lys-C at protease/dehydrogenase ratios of 1:25 for 6 h at 37°C. Amino acids were analyzed in an LKB Alpha Plus instrument after acid hydrolysis at 110°C for 24 h in 6 M HCl/0.5% phenol, while sequencer analysis utilized a Milligen Prosequencer 6600. Nitrotyrosine and PTH-nitrotyrosine were detected by the absorption at 360 nm in acid [1].

3. RESULTS

3.1. *Nitration of native enzymes with tetranitromethane*

3.1.1. Medium-chain dehydrogenases.

Liver alcohol and sorbitol dehydrogenases are inactivated by tetranitromethane, but rates and products are different, with sorbitol dehydrogenase the more sensitive enzyme (90–95% inactivation in 3 min vs. 120 min for alcohol dehydrogenase at enzyme/reagent ratios of 1:20). Acid hydrolysis and analysis for nitrotyrosine after inactivation to 90–95%, revealed about 2 mol nitrotyrosine per mol subunit in alcohol dehydrogenase but only trace amounts in sorbitol dehydrogenase. These results suggest that the most reactive residue in sorbitol dehydrogenase, correlating with inactivation, is not tyrosine. In contrast, no residue in alcohol dehydrogenase is highly sensitive to tetranitromethane, but tyrosine residues gradually react. The presence of a reactive residue in sorbitol dehydrogenase, not correlating with nitration of tyrosine, makes this enzyme suitable for analysis of tyrosine modification after thiol protection.

3.1.2. Short-chain dehydrogenase.

At room temperature, inactivation was similar to that for sorbitol dehydrogenase even at very low tetranitromethane excess. At 4°C, a slower inactivation could be observed, as well as non-stoichiometric amounts of nitrotyrosine after hydrolysis (about 0.5 mol per mol protein chain at 95% inhibition). It is concluded that both tyrosine and other residues react, making tetranitromethane modification complex with *Drosophila* alcohol dehydrogenase.

3.2. *Protection of reactive cysteine residues and subsequent tyrosine modification*

3.2.1. Medium-chain dehydrogenases.

Liver alcohol and sorbitol dehydrogenases were both inactivated by sulphite in the presence of Cu^{2+} (Fig. 1). Subsequent dialysis did not reactivate the enzymes. They were stable to treatment with sulphite alone (Fig. 1), and to treatment with only 0.05 mM cupric sulphate. It is concluded that the reactive cysteine residues of both enzymes are sensitive to sulphation in the presence of Cu^{2+} (the latter necessary for sulphation of thiols [14–16]).

The enzymes inactivated by the sulphite/Cu^{2+} treatment can be reactivated with reducing agents like dithiothreitol at room temperature for 30 min and reach constant values (Fig. 2). Even if incomplete, the reactivations are remarkable, considering that these enzymes

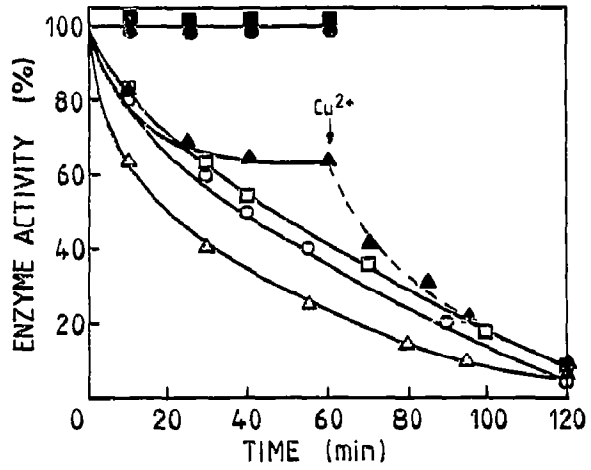

Fig. 1. Inactivation of the three enzymes by sulphite treatment. Open symbols refer to samples treated with 5 mM Na_2SO_3 and 0.05 mM $CuSO_4$ in 0.1 M phosphate buffer, pH 8.0, at room temperature with horse liver alcohol dehydrogenase (□), sheep liver sorbitol dehydrogenase (○) and *Drosophila* alcohol dehydrogenase (△), and filled symbols to samples treated under identical conditions except for the absence of the $CuSO_4$ (until added, at the arrow marked Cu^{2+}).

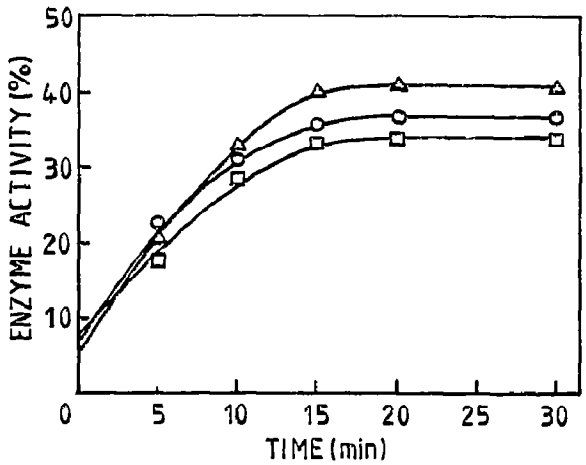

Fig. 2. Reactivation of the three enzymes by dithiothreitol after sulphite treatment. Reactivation of horse liver alcohol dehydrogenase (□), sheep liver sorbitol dehydrogenase (○), and *Drosophila* alcohol dehydrogenase (△) in 0.1 M phosphate buffer, pH 8.0, with 5 mM dithiothreitol at room temperature. Further reactivation was observed after dialysis against phosphate buffer.

are zinc metalloproteins with sensitive apoforms, susceptible to metal exchange, and inhibited by high concentrations of dithiothreitol. Indeed, subsequent dialysis to remove excess reagents was found to carry the reactivation further, but exact conditions for balancing oxidative losses, metal exchanges, and replacements proved difficult to find. Nevertheless, the levels reached show that *S*-sulphocysteine protection is principally reversible even for these Cys-rich proteins.

After sulphite/Cu^{2+} treatment for 2 h at room temperature, at which stage remaining activity was only 5–10% (Fig. 1), and dialysis against 0.1 M phosphate buffer, pH 8.0, the *S*-sulphocysteine-protected enzymes were submitted to treatment with a 20-fold molar excess of tetranitromethane for 5 min at room temperature, i.e. conditions that without protection produce extensive inactivation of sorbitol dehydrogenase. Subsequent cysteine deprotection with 5 mM dithiothreitol yielded a reactivation of alcohol dehydrogenase (64% of that obtained without intervening tetranitromethane treatment), while sorbitol dehydrogenase was permanently inactivated. Apparently, protected sorbitol dehydrogenase has tyrosine residue(s) more accessible than in alcohol dehydrogenase. Absorbance at 428 nm indicated the presence of nitrotyrosine in the tetranitromethane-treated sorbitol dehydrogenase but not in the alcohol dehydrogenase, and amino acid analysis showed the presence of 0.9 nitrotyrosine per subunit for sorbitol dehydrogenase but only trace amounts for alcohol dehydrogenase. These results suggest that one tyrosine residue in sorbitol dehydrogenase is sensitive to tetranitromethane, and that modification of this residue prevents the reactiviations otherwise obtained upon cysteine deprotection (Fig. 2).

3.2.2. Short-chain dehydrogenase.

Treatment of *Drosophila* alcohol dehydrogenase through cysteine protection and deprotection yielded results (Figs. 1 and 2) similar to those with the medium-chain dehydrogenases but with two consistent differences. One is that the *Drosophila* enzyme, with less cysteine and without metal, gives the highest reactivation upon deprotection (Fig. 2). However, even in this case, reactivation is incomplete, and may correlate with additional cysteine modifications (cf. below).

The other is that the *Drosophila* enzyme was sensitive to sulphite both in the presence and absence of Cu^{2+}, although to different extents. In the presence of Cu^{2+}, the enzyme was inactivated in a manner similar to that for the medium-chain dehydrogenases. However, it was not stable to treatment with sulphite alone, and partial inactivation, reaching a plateau, was achieved (Fig. 1), at which stage addition of Cu^{2+} led to further inactivation. Sulphite in the absence of Cu^{2+} can react with cystine [15], and the partial inactivation of the *Drosophila* enzyme under these conditions suggests a non-stoichiometric absence of the two cysteine thiols per subunit in the enzyme preparation. With sulphite alone, the sulphation would cause inactivation in the fraction of the enzyme lacking thiols, and in the presence of Cu^{2+} also in the thiol-containing fraction (Fig. 1). This pattern suggests that the cysteine residues in *Drosophila* alcohol dehydrogenase are not catalytic, but may perturb structural relationships, in agreement with results of site-directed mutagenesis, showing loss of the cysteine residues still to result in active enzymes [18].

Super-reactivation with cyanide of the Cys-protected *Drosophila* alcohol dehydrogenase was noticed. Thiosulphates react readily with aqueous cyanide to give thiocyanates [18]. When *S*-sulphocysteine-protected *Drosophila* enzyme was treated with 10 mM potassium cyanide in 0.1 M phosphate buffer, pH 8.0, for 1 h, and excess reagents were removed by dialysis, enzyme reactivation was found to be 120% of the original value. This result confirms that protein thiols do not play important roles in the enzyme catalysis but that their exact surroundings have structural consequences, influencing overall levels of activity.

It was not possible to reactivate tetranitromethane-treated Cys-protected *Drosophila* alcohol dehydrogenase by cysteine deprotection with 5 mM dithiothreitol or 10 mM potassium cyanide. The modified enzyme exhibited a UV absorbance at 428 nm, and gave 1.8 mol nitrotyrosine per mol subunit after acid hydrolysis. The results show that more than one tyrosine residue in *Drosophila* alcohol dehydrogenase is sensitive to tetranitromethane after cysteine protection.

3.3. *Identification of the labelled tyrosine residues*

Nitrated and carboxymethylated liver sorbitol and *Drosophila* alcohol dehydrogenases, constituting the two enzymes exhibiting reactive tyrosine residues, were digested with proteinase Lys-C, and the peptides obtained were separated by reverse-phase HPLC. Acid hydrolysis and analysis for nitrotyrosine of all major fractions revealed one nitrated peptide in sorbitol dehydrogenase in agreement with the stoichiometry and four in lower yield in *Drosophila* alcohol dehydrogenase (Fig. 3).

Sequence analysis of the fraction nitrated in sorbitol dehydrogenase identified this peptide as the fragment 106–132. The label was identified at cycle 4, corresponding to Tyr-109, and was shown to be PTH-nitrotyrosine both by its absorbance at 360 nm (possible to monitor in the MilliGen Prosequencer directly) and late position in the chromatogram (after PTH-valine). The second tyrosine residue, Tyr-131, was recovered completely unlabelled. This result identifies Tyr-109 as selectively labelled, accessible in sorbitol dehydrogenase, and detectable after cysteine protection.

Similar analysis of the *Drosophila* enzyme showed the nitrated fractions to correspond to peptides with variable recovery of nitrotyrosine at positions 61 and 63 (in peptide 56–74), 147 and 152 (in peptide 144–156), and

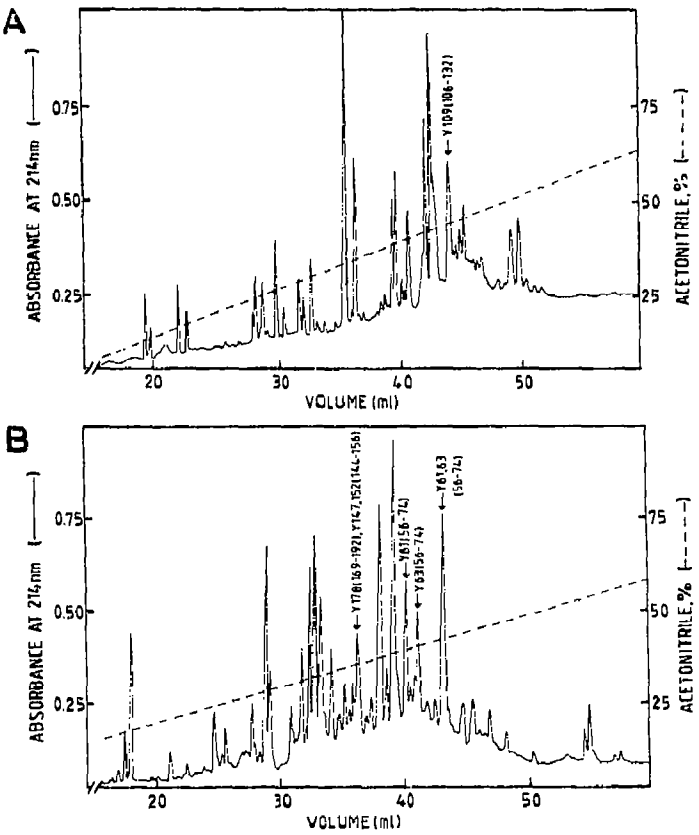

Fig. 3. Purification of the nitrotyrosine-containing peptides from digests of nitrated sorbitol dehydrogenase (A) and *Drosophila* alcohol dehydrogenase (B) by reverse-phase high-performance liquid chromatography on Vydac C18. Elution positions of all nitrotyrosine-containing peptides, with numbers giving the tyrosine position and the sizes of the fragments, as derived from sequence analysis. In the case of fragment 56–74, containing Tyr-61 and Tyr-63 in B, peptide identification and tyrosine positions were further checked by redigestion with endoprotease Asp-N; this fragment was recovered in multiple form, labelled on Tyr-61, Tyr-63, both Tyr, or none.

178 (in peptide 169–192). These labels add up to about two residues of nitrotyrosine and indicate absence of selectivity in the tyrosine reactivity.

4. DISCUSSION

The results have three implications. The first is that the old method of sulphite/Cu^{2+} modification of cysteine residues is appropriate for reversible cysteine modification during tests for reactive tyrosine residues in proteins. *S*-Sulphocysteine derivatization inactivates all these enzymes, and the inactivations are reversible by dithiothreitol treatment. True, reversibility is incomplete under the present conditions, but this is compatible with the fact that two of the enzymes are sensitive metalloproteins (the middle-chain dehydrogenases), and one (the short-chain dehydrogenase) exhibits a heterogeneity in Cys status (Fig. 1). The reactivation achieved was unexpected, and the medium-chain dehydrogenases have not been reversibly activated after other cysteine modifications. Reaction of the cysteine-protected enzymes with tetranitromethane shows that differential tyrosine reactivity is possible to obtain in the protected enzymes. The results exemplify three different situations, one accessible tyrosine residue (sorbitol dehydrogenase), several partly accessible (*Drosophila* alcohol dehydrogenase), and absence of reactive tyrosine residues (liver alcohol dehydrogenase). Since the reaction scheme is possible to use with cysteine-rich dehydrogenases, it should be applicable to proteins in general for detection of reactive tyrosine residues.

The second implication concerns the reactive tyrosine residues identified. They correlate with known conformational properties. In liver alcohol dehydrogenase, no tyrosine residue has been ascribed specific roles at the active site [12], while in sorbitol dehydrogenase, Tyr-109 corresponds to a segment containing the largest differences between the liver alcohol and sorbitol dehydrogenases, adjacent to a deletion, to loss of a zinc atom, and contributing to differences in quaternary structure [13]. Regarding the labelling results with the *Drosophila* enzyme, the tertiary structure is unknown. Only one short-chain dehydrogenase has been analyzed crystallographically [20]. However, that enzyme, 3α/20β-hydroxysteroid dehydrogenase has one Tyr residue, Tyr-152, ascribed a critical role [3], located close to the substrate site [20], and the corresponding residue has been proven to have a role from site-directed mutagenesis of the homologous 15-hydroxyprostaglandin dehydrogenase [21]. Even if the multiplicity of labelling presently obtained prevents exact correlations, the results at least show that the corresponding residue is one of those labelled.

The third implication concerns the special hybrid properties of the *Drosophila* enzyme towards sulphite inactivation in the presence and absence of cupric ions (Fig. 1). In the case of the medium-chain dehydrogenases, both were intensitive towards the sulphite treatment in the absence of Cu^{2+}, confirming the need for Cu^{2+} to get sulphite reaction with free thiols [14–16]. However, in the case of the *Drosophila* enzyme, a substantial inactivation occurred also in the absence of Cu^{2+}, conditions at which the reagent reacts only with disulphide structures [15]. It may be deduced that in the *Drosophila* enzyme one or both of the cysteine residues are linked to other thiols in disulphide arrangements, or to adducts in reactive linkages. Inter-subunit disulphide bridges are not likely to be present, since dimers are not visible upon non-reductive SDS-polyacrylamide gel electrophoresis. An intra-subunit disulphide bridge does also not seem likely since the two Cys positions (135 and 218 in *Drosophila* alcohol dehydrogenase) do not appear easy to perturb into an S–S bridge if the *Drosophila* enzyme has a tertiary structure related to that of 3α/20β-hydroxysteroid dehydrogenase [20]. Therefore, the reactivity in the absence of Cu^{2+} may suggest an unexpected presence of *S*-linked low molecu-

lar weight ligands. The presence of unusual Cys-modifications in the intact enzyme is also supported by the fact that cyanide treatment and subsequent reduction was now found to cause super-activation of the Cys-protected enzyme. Interestingly, the *Drosophila* enzyme has since long been ascribed different molecular forms presumably caused by adduct formation [22,23]. In conclusion, the results demonstrate the use of tetranitromethane-linked tyrosine modification in Cys-rich proteins, establish a specific difference between dehydrogenases, and identify an unexpected activation of the *Drosophila* enzyme.

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REFERENCES

[1] Riordan, J.F. and Vallee, B.L. (1972) Methods Enzymol. 25, 515–521.
[2] Jörnvall, H., Persson, M. and Jeffery, J. (1981) Proc. Natl. Acad. Sci. USA 78, 4226–4230.
[3] Persson, B., Krook, M. and Jörnvall, H. (1991) Eur. J. Biochem. 200, 537–543.
[4] Krook, M., Marekov, L. and Jörnvall, H. (1990) Biochemistry 29, 738–743.
[5] Krook, M., Prozorovski, V., Atrian, S., Gonzàlez-Duarte, R. and Jörnvall, H. (1992) Biochemistry, submitted.
[6] Harris, I. (1964) Nature 203, 30–34.
[7] Li, T.-K. and Vallee, B.L. (1964) Biochemistry 3, 869–873.
[8] Zeppezauer, E., Jörnvall, H. and Ohlsson, I. (1975) Eur. J. Biochem. 58, 95–104.
[9] Jeffery, J., Cummins, L., Carlquist, M. and Jörnvall, H. (1981) Eur. J. Biochem. 120, 229–234.
[10] Bosron, W.F., Yin, S.-J., Dvulet, F.E. and Li, T.-K. (1986) Biochemistry 25, 1876–1881.
[11] Johansson, J., Vallee, B.L. and Jörnvall, H. (1991) FEBS Lett. 279, 119–122.
[12] Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Söderberg, B.-O., Tapia, O., Brändén, C.-I. and Åkeson, Å. (1976) J. Mol. Biol. 102, 27–59.
[13] Eklund, H., Horjales, E., Jörnvall, H., Brändén, C.-I. and Jeffery, J. (1985) Biochemistry 23, 8005–8012.
[14] Clarke, H.T. (1932) J. Biol. Chem. 97, 235–248.
[15] Swan, J.M. (1957) Nature 180, 643–645.
[16] Bailey, J.L. and Cole, R.D. (1959) J. Biol. Chem. 234, 1733–1739.
[17] Ribas de Pouplana, L.P., Atrian, S., Gonzàlez-Duarte, R., Fothergill-Gilmore, L., Kelly, S.M. and Price, N.C. (1991) Biochem. J. 276, 433–438.
[18] Chen, Z., Lu, L., Shirley, M., Lee, W.R. and Chang, S.H. (1990) Biochemistry 29, 1112–1118.
[19] Vanaman, T.C. and Stark, G.R. (1970) J. Biol. Chem. 14, 3565–3573.
[20] Ghosh, D., Weeks, C.M., Grochulski, P., Duax, W.L., Erman, M., Rimsay, R.L. and Orr, J.C. (1991) Proc. Natl. Acad. Sci. USA 88, 10064–10068.
[21] Ensor, C.M. and Tai, H.H. (1991) Biochem. Biophys. Res. Commun. 176, 840–845.
[22] Ursprung, H. and Carlin, L. (1968) Ann. N.Y. Acad. Sci. 151, 456–475.
[23] Winberg, J.-O. and McKinley-McKee, J.S. (1988) Biochem. J. 251, 223–227.