

Unique molecular properties of superoxide dismutase from teleost fish skin

Toshiki Nakano*, Minoru Sato, Masaaki Takeuchi

Marine Biochemistry Laboratory, Faculty of Agriculture, Tohoku University, Aoba-ku, Sendai 981, Japan

Received 3 January 1995

Abstract A unique Cu,Zn-SOD was found and isolated from plaice *Paralichthys olivaceus* skin. Surprisingly, the properties of purified fish skin SOD were very different from those of SOD from other sources reported so far. The purified SOD was composed of four same subunits of 16 kDa and the molecular weight of the native SOD was found to be around 65 kDa. The dominant amino acids of the SOD were Ser, Thr, Pro and Glu. Above 70°C, thermostability of the SOD was much lower than that of bovine erythrocyte Cu,Zn-SOD.

Key words: Superoxide dismutase; Molecular properties; Thermostability; Teleost; *Paralichthys olivaceus*; Fish skin

1. Introduction

Aerobic organisms have both chemical and enzymatic defence systems against the toxicity caused by reactive oxygen species (ROS) [1]. The antioxidant enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), might contribute to the defence systems. There are three known types of SODs depending upon the metals found in their active sites: copper/zinc (Cu,Zn-SOD), manganese (Mn-SOD) and iron (Fe-SOD). Cu,Zn-SOD is found predominantly in the cytosolic fraction and is normally associated with eukaryotes, and shows very sensitive behavior to cyanide and H₂O₂. Mn-SOD is associated with mitochondria or prokaryotes, and is insensitive to cyanide. Fe-SOD is found in prokaryotes and in a few families of plants, and is not sensitive to cyanide but is inhibited by H₂O₂ [2,3]. Furthermore, an extracellular form of Cu,Zn-SOD, which is called EC-SOD, is distinct from the cytosolic Cu,Zn-SOD, is also found in eukaryotes [4]. Recently, in the course of the studies on antioxidant defence systems of fish [5,6], we have detected Cu,Zn-SOD and Mn-SOD activities in fish skin, and these SODs activities were observed to be higher in the dark than in the light parts of skin [7]. ROS is considered to be correlated with melanization in mammalian skin [8,9]. The dark parts of fish skin are also supposed to contain melanophores, in which melanization should occur [10]. Hence, the distribution of higher SODs activities in the dark parts of fish skin might be related to melanization and to regulation of ROS. Fish SODs were reported to exhibit a large variability of the isoelectric point (pI) value over a wide pH range and often have an unusually high pI value [11]. Thereby, fish SOD has a possibility to show unusual and novel properties. However, no report on the relationship between SODs, ROS and melanization in fish skin is available. In the present investigation, we have purified a Cu,Zn-SOD from the

dark parts of skin of plaice *Paralichthys olivaceus* and described unique properties of this enzyme.

2. Experimental

2.1. Materials

Cytochrome *c* (cyt. *c*) from horse heart and bovine erythrocyte Cu,Zn-SOD were obtained from Sigma. All other chemicals were of analytical grade. Live specimens of plaice *P. olivaceus* were donated by Fukushima Prefectural Fish Farming Experimental Station, Fukushima, Japan.

2.2. Purification of the Cu,Zn-SOD electromorphs from plaice skin

All the procedures were carried out at 3–5°C. Samples of plaice were killed, brought to the laboratory in an ice box, and dark parts of the skin tissue were carefully collected. The skin was thoroughly washed with sterilized ice-cold saline solution (0.9% w/v) and then homogenized in 7 mM Tris-HCl buffer (pH 7.1) (buffer A). The homogenate was centrifuged at 30 000 × *g* for 20 min, and the supernatant was dialyzed against the buffer A. The dialyzed solution was applied to a DEAE-Toyopearl column equilibrated with the buffer A. Non-adsorbed active fractions were collected and dialyzed against 7 mM KH₂PO₄/Na₂HPO₄ buffer (pH 5.1) (buffer B). The dialyzed solution was loaded onto a SP-Toyopearl column equilibrated with the buffer B. After washing with the buffer B, the adsorbed proteins were eluted with a linear gradient from 0 to 1.0 M KCl in the buffer B and the active fractions were pooled and dialyzed against the buffer A.

2.3. Polyacrylamide gel electrophoresis (PAGE)

Native PAGE was carried out in 6.5% acrylamide slab gels at 4°C [12]. Sodium dodecyl sulfate (SDS)/PAGE was performed in 10% gels at room temperature [13]. After electrophoresis, the gels were stained with Bio-Rad Silver stain Kit (Bio-Rad Laboratories, USA) for protein detection.

2.4. Enzyme activity assays

SOD activity was measured by two types of assay methods as follows: (i) Spectrophotometric method was based on the inhibition of xanthine/xanthine oxidase induced reduction of cyt. *c* in 50 mM phosphate buffer, pH 7.8, at 25°C [14]. The assay was run at least in triplicate and the mean values were reported. One unit of activity was defined as the amount of protein required to inhibit the rate of reduction of cyt. *c* by 50% under the assay conditions given above. (ii) Visualization of the SOD activity on native PAGE gels was performed utilising the nitroblue tetrazolium illumination system as described previously [15].

2.5. Amino acid (AA) composition analysis

Samples of purified SOD-Fs were dialyzed against distilled water. AA compositions were determined with a Jasco HPLC-800 AA analyzing system (Nihon Bunko Co., Ltd., Japan) after hydrolysis with 6 N HCl under vacuum at 108°C for 24 h.

3. Results

3.1. Purification of fish skin Cu,Zn-SOD (SOD-Fs)

The results of a typical purification are summarized in Table 1. It is important to show that the purified fish-skin enzyme is a real SOD and not a copper-containing protein such as ceruloplasmin. SOD is known to scavenge superoxide radical

*Corresponding author. Fax: (81) (22) 272 1870.

Abbreviation: SOD-Fs, fish skin Cu,Zn-SOD.

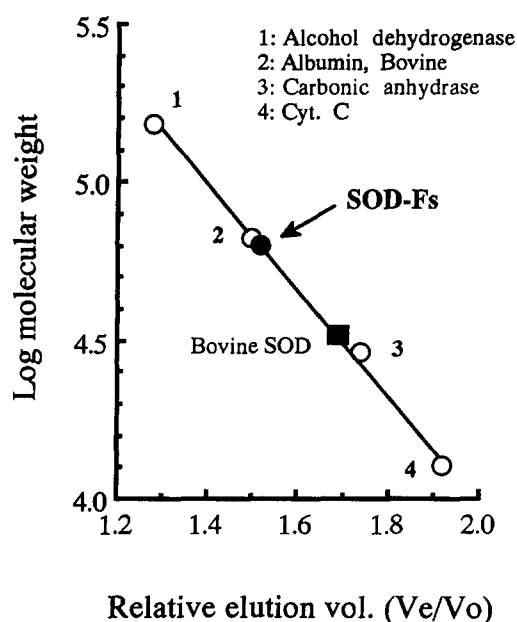


Fig. 1. Determination of native molecular weights of fish skin SOD (SOD-Fs) and bovine erythrocyte SOD by Superose 12 HR column chromatography; V_e , elution volume; V_o , void volume.

(O_2^-) and to accelerate dismutation of the radical to O_2 and H_2O_2 [14,16]. For the determination of enzyme-induced accelerated H_2O_2 formation, H_2O_2 concentration of enzyme reaction solution was measured by the modified scopoletin method [17]. In practice, when the enzyme reaction was carried out at higher O_2^- concentration compared with the normal assay conditions [14], the fish enzyme and bovine SOD were observed to accelerate H_2O_2 production. The H_2O_2 production rates were obtained to be $2.01 \mu M/min$ for 48 nM fish enzyme, $1.53 \mu M/min$ for 20 nM bovine SOD, and $0.98 \mu M/min$ for the control (spontaneous disproportionation of O_2^-). These results suggest that the fish enzyme and bovine SOD act in similar fashion. On the other hand, for example, ceruloplasmin produces an increase in H_2O_2 production [18] and is thought not to dismute O_2^- but to react stoichiometrically with O_2^- [19]. In addition, copper proteins, such as ceruloplasmin and metallothionein, need much higher amount of protein than SOD when they scavenge O_2^- [18,20]. Thus, the purified fish skin enzyme is considered to be a SOD. The identical electrophoretic mobility of purified fish skin SOD, which was named SOD-Fs, and the low mobility activity zone in the crude extract demonstrates that no significant modification of the protein occurred during the purification. The visualized SOD activity of the gel disappeared in the presence of KCN. Furthermore, SOD activity decreased in proportion to the KCN and H_2O_2 concentrations (data not shown). These phenomena suggest that SOD-Fs is very sensi-

tive to cyanide and H_2O_2 , and is thought to contain copper and zinc in its active sites. According to recent findings, there were two distinct Cu,Zn-SOD electromorphs in plaice skin [7]. We concluded, therefore, that the SOD-Fs obtained by the two ion-exchange chromatographies was one of the Cu,Zn-SOD electromorphs of skin.

3.2. Molecular properties of the purified SOD-Fs

The apparent molecular weight (MW) of the native SOD-Fs was found to be around 65 kDa from the gel filtration on a calibrated Superose 12 column (Fig. 1). On SDS/PAGE, SOD-Fs gave a single protein band with apparent mol.wt. of 16 kDa regardless of the presence of 2-mercaptoethanol (2-ME) (Fig. 2). No other bands were visible on the gels. Surprisingly, the apparent mol.wt. data from the gel filtration profile and SDS/PAGE indicates that SOD-Fs is composed of four subunits of equal size held together by non-covalent interactions and not by disulfide (S-S) bridges. On the other hand, the apparent mol.wt. of native bovine SOD was observed to be around 33 kDa from the gel filtration (Fig. 1). Furthermore, upon SDS/PAGE, bovine SOD appeared to have the same subunit mol.wt. of 16 kDa in the presence of 2-ME, and the apparent mol.wt. was approximately 32 kDa in the absence of 2-ME (Fig. 2). These observations suggest that bovine SOD is of dimeric structure with two identical subunits and that disulfide bridges seem to contribute to the stability of the holoenzyme.

3.3. AA composition

In comparison with the AA compositions reported Cu,Zn-SODs from all other sources, SOD-Fs exhibits differences (Fig. 3). The most dominant AA of SOD-Fs were Ser, followed by Thr, Pro and Glu. On the other hand, the most predominant AA of SODs from other sources were Gly, followed by Asp, Val and Glu. SOD-Fs was in good agreements with other sources in the low contents of aromatic AA, such as Tyr and Phe. Thus, SOD-Fs seems to be abundant in polar AA, compared to other sources.

3.4. Thermal stability

When the enzymes were incubated for 1 h at different temperatures from 30 to 70°C, the activities of SOD-Fs and bovine SOD remained at the original level of below 40°C (Fig. 4). However, an abrupt decline in activity of SOD-Fs occurred above 60°C. Heating at 70°C, SOD-Fs was completely inactivated, while bovine SOD retained about 80% of the original activity.

4. Discussion

The Cu,Zn-SOD is known to be one of the significantly conserved families of enzymes, with regard to its structural

Table 1
Purification of fish skin Cu,Zn-SOD (SOD-Fs)^a

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Crude extract	412.0	1,524.4	3.7	100	1
DEAE-Toyopearl	30.16	838.4	27.8	55.0	8
SP-Toyopearl	0.02	22.2	1,111.1	1.5	300

^a 50 g of fish (plaice) skin was used for enzyme purification. See section 2 for details.

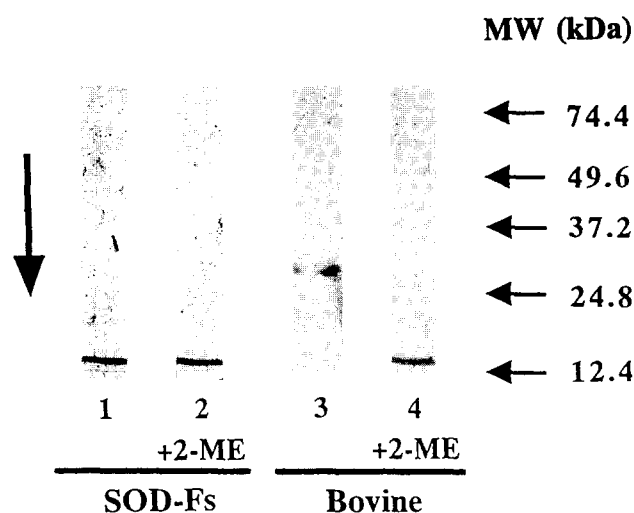


Fig. 2. SDS/PAGE of fish skin SOD (SOD-Fs) (1 and 2) and bovine erythrocyte SOD (3 and 4). The arrows indicate the positions of the molecular weight markers.

properties. Generally speaking, most of the purified Cu,Zn-SODs reported so far, have been shown to have a mol.wt. of 32 kDa, a subunit mol.wt. of 16 kDa, and to be a dimer composed of identical subunits [21,22]. Hence, SOD-Fs which appears to be of tetrameric structure as the native form, is very interesting. In addition, SOD-Fs was able to be dissociated into subunits with SDS even in the absence of 2-ME. Most of Cu,Zn-SODs are reported not to be able to dissociate into subunit in the absence of 2-ME. Actually, in bovine SOD our results were in good agreement with the published data (Figs. 1 and 2) [23,24]. The subunits of bovine SOD are thought to be associated through unusually strong non-covalent interactions so that SOD is not unfolded by SDS. The disulfide reducing agents, such as 2-ME, appear to act on disulfide bridges localized within each subunit of bovine SOD, result in initial conformational changes of the protein and, consequently, in a total disruption of the protein molecule into subunits [23,24]. Thus,

the subunit interactions seem to be different between in the SOD-Fs and in the bovine SOD. A similar phenomenon is also observed with swordfish liver [25] and aquatic bacterium [26]. On the other hand, EC-SOD is known to have a high mol.wt. (135 kDa) so that SOD-Fs seems to be different from EC-SOD [4]. When SOD is incubated under denaturing conditions, formation of aggregates (mol.wt. 91–201 kDa) are observed [25,27]. A similar observation is obtained from undissociated bovine SOD and from old prepared pig liver SOD [23,28]. However, in this study, gel filtration was carried out under native and low temperature conditions. Furthermore, freshly prepared SOD-Fs samples had been used. Consequently, the aggregation or undissociation of SOD-Fs are supposed to be unlikely to occur.

The AA composition of SOD-Fs is clearly different from those of human EC-SOD [4], human Mn-SOD [29] and *E. coli* Fe-SOD [30]. We also found that immobilized-metal-affinity column could adsorb not SOD-Fs but bovine erythrocyte SOD (data not shown). This affinity column is also known to adsorb human erythrocyte SOD [31]. The heavy metals in the affinity columns are thought to be attached to certain kinds of AA residues at the protein surface [32]. Consequently, the AA residues which are located on the SOD-Fs surface, may be different from those of bovine and human SODs.

In general, Cu,Zn-SOD is known to have a very stable three-dimensional structure and to show unusually high thermal stability [33,34]. In comparison with SOD-Fs, AA compositions of SODs from other sources, are rich in hydrophobic AAs (e.g. Val and Ile) (Fig. 3). It is suggested that hydrophobicity of a protein may play an especially important role in its thermal stability [35,36]. On the other hand, polar AA residues, such as Ser and Thr, were undesirable for establishing thermal stability in protein [36]. Therefore, one of the reasons for the low thermal stability of SOD-Fs, seems to be concerned with low amounts of hydrophobic AAs, high amounts of polar AAs and the strength of non-covalent interactions between the subunits of SOD-Fs. Non-covalent interactions, such as electrostatic interactions and hydrogen bonds, are also thought to contribute to protein stability and flexibility when they occur on the surface of proteins [37,38]. Furthermore, it is also pointed out

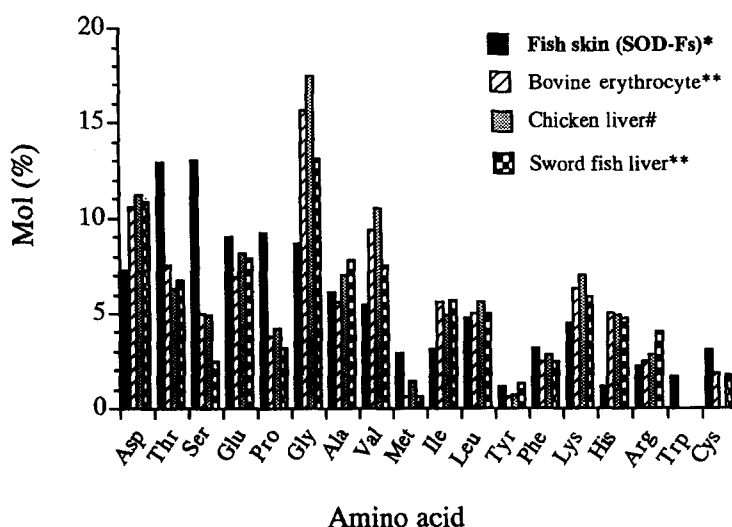


Fig. 3. Comparison of amino acid compositions of Cu,Zn-SODs isolated from different sources (mol %). The percentages of Trp and Cys of chicken liver SOD were not determined. *This work; **Bannister et al. [25]; #Weisiger and Fridovich [48].

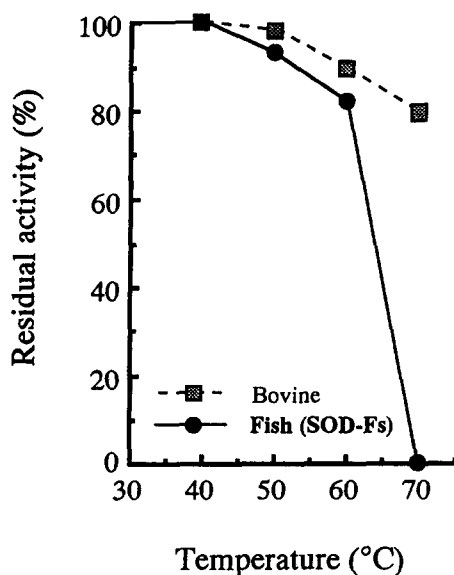


Fig. 4. Thermal stability of SOD activity from fish skin and bovine erythrocyte. The enzymes in 50 mM sodium phosphate buffer (pH 7.8), were incubated at different temperatures for 1 h. After incubation, the enzymes were immediately cooled in ice water, and then measured for the residual activity at 25°C. Prior to addition of the enzymes, tubes were pre-incubated for a few minutes at respective temperatures. The concentrations of fish and bovine enzymes were 0.08 and 0.04 mg protein/ml, respectively.

that the number and positions of free Cys residues in Cu,Zn-SOD affect enzyme stability [33,39]. In any event, detailed structural data of SOD-Fs is desirable to get a clear answer for the thermal instability of SOD-Fs.

In conclusion, this report demonstrates for the first time the existence of unique SOD in fish skin. In recent studies, red tide unicellular phytoplankton *Chattonella marina* which often causes fish death, is reported to generate ROS [40,41]. Furthermore, in the sea, ROS are reported to be produced photochemically [42]. Fish skin contains oxygen-sensitive substances such as polyunsaturated fatty acids. Under these conditions, fish skin has many opportunities to be under oxidative stress. As a result, SOD of fish skin may be important in the defence system of skin against ROS stress. The molecular properties of SOD-Fs differ from those of Cu,Zn-SODs isolated from other sources including some bacteria, such as *Photobacter leiognathi* which is known to live symbiotically with a certain marine fish [12] and free-living, non-symbiotic bacterium [26]. We also observed that SOD-Fs had pI about 6.3 on isoelectrofocusing gel (data not shown), in contrast to the acidic values of most mammalian Cu,Zn-SODs [26]. However, it is not yet clear why fish skin has unique SOD. Recently, it is observed that the SOD mutations identified in certain patients influence both the active site and the structure of SOD and finally the stability of SOD is dramatically disrupted [43–45]. Hence, there is a possibility that some mutations also affect fish skin SOD. Some microorganisms are known to contain unique forms of SOD such as Fe,Mn,Zn-SOD [46,47]. Unfortunately, the metal contents of SOD-Fs was not determined because insufficient enzyme was obtained. Further investigations (e.g. metal contents, primary structure etc.) are now in progress.

Acknowledgements: We thank Mr. T. Suzuki of Applied Technology R & D Center, Tohoku Electric Power Co. Inc., Japan, for his technical assistance. We also wish to thank Dr. T. Watanabe, Professor Emeritus at the University of British Columbia, Canada, for reading of the manuscript. This study was supported in part by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan for T. Nakano.

References

- [1] Asada, K. (1978) Protein, Nucleic Acid, and Enzyme (Tokyo) 23, 200–213.
- [2] Asada, K. (1976) in: Method of Plant Enzyme and Protein (Y. Morita, M. Shin, K. Asada and S. Ida, Eds.) pp. 373–378. Kyoritsu Press, Tokyo.
- [3] Asada, K., Kanematsu, S., Okada, S. and Hayakawa, T. (1980) in: Chemical and Biological Aspects of Superoxide and Superoxide Dismutase (J.V. Bannister and H.A.O. Hill, Eds.) pp. 136–153. Elsevier, Amsterdam.
- [4] Marklund, S.L. (1982) Proc. Natl. Acad. Sci. USA 79, 7634–7638.
- [5] Nakano, T., Sato, M. and Takeuchi, M. (1992) Comp. Biochem. Physiol. 102B, 31–35.
- [6] Nakano, T., Sato, M. and Takeuchi, M. (1992) J. Food Sci. 57, 1116–1119.
- [7] Nakano, T., Sato, M. and Takeuchi, M. (1993) J. Fish Biol. 43, 492–496.
- [8] Joshi, P.C., Carraro, C. and Pathak, M.A. (1987) Biochem. Biophys. Res. Commun. 142, 265–274.
- [9] Oyanagui, Y. (1990) Fragrance, J. (Tokyo) 18, 94–97.
- [10] Matsumoto, J. (1990) Heredity (Tokyo) 44, 31–35.
- [11] Martin, J.P. and Fridovich, I. (1981) J. Biol. Chem. 256, 6080–6089.
- [12] Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427.
- [13] Laemmli, U.K. (1970) Nature 227, 680–685.
- [14] McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
- [15] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
- [16] Weser, U. and Voelcker, G. (1972) FEBS Lett. 22, 15–18.
- [17] Andrae, W.A. (1955) Nature 175, 859–860.
- [18] Goldstein, I.M., Kaplan, H.B., Edelson, H.S. and Weissmann, G. (1979) J. Biol. Chem. 254, 4040–4045.
- [19] Bannister, J.V., Bannister, W.H., Hill, H.A.O., Mahood, J.F., Willson, R.L. and Wolfenden, B.S. (1980) FEBS Lett. 118, 127–129.
- [20] Shiraishi, N., Morimoto, S., Joja, I., Iida, S., Aono, K. and Utsumi, K. (1983) Igakuno Ayumi (Tokyo) 126, 245–247.
- [21] Steinman, H.M. (1982) in: Superoxide Dismutase (L.W. Oberley, Ed.) Vol. 1, pp. 11–68. CRC Press, FL.
- [22] Kitagawa, Y. and Katsube, Y. (1988) Protein, Nucleic Acid, and Enzyme (Tokyo) 33, 231–236.
- [23] Abernethy, J.L., Steinman, H.M. and Hill, R.L. (1974) J. Biol. Chem. 249, 7339–7347.
- [24] Fridovich, I. (1975) Annu. Rev. Biochem. 44, 147–159.
- [25] Bannister, J.V., Anastasi, A. and Bannister, W.H. (1977) Comp. Biochem. Physiol. 56B, 235–238.
- [26] Steinman, H.M. (1982) J. Biol. Chem. 257, 10283–10293.
- [27] Bartkowiak, A., Leyko, W. and Fried, R. (1979) Comp. Biochem. Physiol. 62B, 61–66.
- [28] Salin, M.L. and Wilson, W.W. (1981) Mol. Cell. Biochem. 36, 157–161.
- [29] McCord, J.M., Boyle, J.A., Day, E.D., Rizzolo, L.J. and Salin, M.L. (1977) in: Superoxide and Superoxide Dismutase (A.M. Michelson, J.M. McCord and I. Fridovich, Eds.) pp. 129–138. Academic Press, London.
- [30] Asada, K., Kanematsu, S. and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243–256.
- [31] Miyata-Asano, M., Ito, K., Ikeda, H., Sekiguchi, S., Arai, K. and Taniguchi, N. (1986) J. Chromatogr. 370, 501–507.
- [32] Kasai, K. (1991) in: Affinity Chromatography (K. Kasai, I. Matsumoto and M. Beppu, Eds.) pp. 82–83. Tokyo Kagaku Dozin, Tokyo.

- [33] Tainer, J.A., Getzoff, E.D., Beem, K.M., Richardson, J.S. and Richardson, D.C. (1982) *J. Mol. Biol.* 160, 181–217.
- [34] Roe, J.A., Butler, A., Scholler, D.M. and Valentine, J.S. (1988) *Biochemistry* 27, 950–958.
- [35] Bigelow, C.C. (1967) *J. Theoret. Biol.* 16, 187–211.
- [36] Mozhaev, V.V. and Martinek, K. (1984) *Enzyme Microb. Technol.* 6, 50–59.
- [37] Mrabet, N.T., van den Broeck, A., van den Brande, I., Stanssens, P., Laroche, Y., Lambeir, A.-M., Matthijssens, G., Jenkins, J., Chiadmi, M., van Tilbeurgh, H., Rey, F., Janin, J., Quax, W.J., Lasters, I., de Mayer, M. and Wodak, S.J. (1992) *Biochemistry* 31, 2239–2253.
- [38] Serrano, L., Horovitz, A., Avron, B., Bycroft, M. and Fersht, A.R. (1990) *Biochemistry* 29, 9343–9352.
- [39] Hallewell, R.A., Imlay, K.C., Lee, P., Fong, N.M., Gallegos, C., Getzoff, E.D., Tainer, J.A., Cabelli, D.E., Tekamp-Olson, P., Mullenbach, G.T. and Cousens, L. (1991) *Biochem. Biophys. Res. Commun.* 181, 474–480.
- [40] Oda, T., Ishimatsu, A., Shimada, M., Takeshita, S. and Muramatsu, T. (1992) *Marine Biol.* 112, 505–509.
- [41] Oda, T., Akaike, T., Sato, K., Ishimatsu, A., Takeshita, S., Muramatsu, T. and Maeda, H. (1992) *Arch. Biochem. Biophys.* 294, 38–43.
- [42] Mopper, K. and Zhou, X. (1990) *Science* 250, 661–664.
- [43] Deng, H.-X., Hentati, A., Tainer, J.A., Iqbal, Z., Cayabyab, A., Hung, W.-Y., Getzoff, E.D., Hu, P., Herzfeldt, B., Roos, R.P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H.E., Ahmed, A., Roses, A.D., Hallewell, R.A., Pericak-Vance, M.A. and Siddique, T. (1993) *Science* 261, 1047–1051.
- [44] Aoki, M., Ogasawara, M., Matsubara, Y., Narisawa, K., Nakamura, S., Itoyama, Y. and Abe, K. (1993) *Nature Genetics* 5, 323–324.
- [45] Nakano, R., Sato, S., Inuzuka, T., Sakimura, K., Mishina, M., Takahashi, H., Ikuta, F., Honma, Y., Fujii, J., Taniguchi, N. and Tsuji, S. (1994) *Biochem. Biophys. Res. Commun.* 200, 695–703.
- [46] Beaman, B.L., Scates, S.M., Moring, S.E., Deem, R. and Misra, H.P. (1983) *J. Biol. Chem.* 258, 91–96.
- [47] Searcy, K.B. and Searcy, D.G. (1981) *Biochim. Biophys. Acta* 670, 39–46.
- [48] Weisiger, R.A. and Fridovich, I. (1973) *J. Biol. Chem.* 248, 3582–3592.