injections of $CdCl_2$, $BiCl_3$ or $HgCl_2$, respectively [8]. Equine metallothionein was isolated from horse kidney [9]. Optical measurements were carried out as previously reported [2]. Figure 1 shows an example of the optical changes that occur when mercury is added *in vitro* to metallothionein. In these experiments horse kidney Cd,Zn-MT 1 was titrated with aliquots of an aqueous solution of $Hg(NO_3)_2$. The metal concentrations in the Cd, Zn-MT 1 were determined by atomic absorption spectroscopy; the values measured were Cd: 4.0, Zn: 1,2 and Cu: 0.2 (each expressed as moles of metal/mole of protein).

Line #1 in Fig. 1 shows the spectra obtained by all three techniques for the native protein; it is characteristic of Cd,Zn-MT data that derivative-shaped CD and MCD envelopes are observed under the 250 nm shoulder in the absorption spectrum [2, 3]. As mercury is slowly added, we observe first a slight increase in absorbance at 250 nm, however, after the addition of 1.50 mol eq (line #5) the absorbance at 250 nm gradually decreases. Throughout the whole titration the absorbance at 300 nm, and both the CD and MCD spectra show continuous changes that mark the loss of the cadmium and the binding of mercury to the protein. The final traces in Fig. 1 (line #9) show that the cadmium has been displaced by the mercury: there is no longer a shoulder at 250 nm in the absorption spectrum, and the derivative-shaped envelopes in both CD and MCD spectra have collapsed. The new absorption, CD and MCD intensity at 300 nm is assigned as the sulfur to mercury charge transfer band. We observe similar spectra for the Hg,Cu-MT from rat kidney and Hg complexes of BAL that serve as models of the binding sites in metallothioneins.

Acknowledgements. We thank the NSERC of Canada for financial support.

1 J. H. R. Kägi and M. Nordberg, *Experientia Supp.*, 34 (1979).
2 A. Y. C. Law and M. J. Stillman, *Biochem. Biophys. Res. Commun., 102,* 397 (1981).
3 U. Weser and H. Rupp 'The Chemistry, Biochemistry and biology of Cadmium', Ch. 7, M. Webb, ed., Elsevier/North Holland Biochemical Press, Amsterdam, 1979.
4 J. D. Otvos and I. M. Armitage, 'Biochemical Structure Determination by NMR', Ch. 4, B. D. Sykes, J. Glickson, A. A. Bothner-By, eds., Dekker, New York, 1981.
5 A. J. Żelazowski and J. K. Piotrowski, *Biochim. Biophys. Acta, 625,* 89 (1980).
6 M. Vašák, J. H. R. Kägi and H. A. O. Hill, *Biochemistry, 20,* 2852 (1981).
7 G. Sokolowski, W. Pilz and U. Weser, *FEBS-Letters, 48,* 222 (1974).
8 A. J. Żelazowski, J. A. Szymańska and H. Witas, *Prep. Biochem., 10,* 95 (1980).
9 A. J. Żelazowski, J. K. Piotrowski, E. M. Mogilnicka, J. A. Szymańska and B. W. Kaszper, *Bromat. Chem. Toksykol., 11,* 51 (1978).

O13

Isolation of Three Peptidases from *Bacillus subtilis* 168 ind$^-$ with Different Specificity and Metal-Ion Activation

R. K. VAITKEVIČIUS and G. B. BABARSKIENĖ

All-Union Research Institute of Applied Enzymology, Vilnius, Lithuanian SSR, U.S.S.R.

Three metal-dependent peptidases (I, II, III) were isolated from the cells of *B. subtilis* 168 ind$^-$. Phosphate buffers of pH 6.5 were used for all purification steps. Precautions were taken to avoid the contamination of enzymes by heavy metal-ions [1] in the course of the purification procedure. I and III were separated from II on DE-52 cellulose by salt gradient and I from III on hydroxylapatite with phosphate buffer concentration gradient. In addition all 3 enzymes were purified subsequently on Sephadex G-150 and DE-52 cellulose.

Leu-β-NA, Leu-Gly-Gly and Val-Val were used as specific substrates for I, II and III respectively after activation by selected metal-ions. All 3 enzymes are metalloenzymes with a comparatively weak metal-ion–enzyme bonding, thus the enzymes were isolated as inactive apoenzymes. The molecular weights of these apoenzymes are 52000, 87000 and 44000 (by gel-chromatography), isoelectric points 4.9, 4.4 and 4.7.

The mutually-independent pH-metal-ion buffers on the basis of diethylbarbiturate (phosphate or tris in some cases) for pH fixation and citrate for fixation of metal-ion concentration were used for activity determination in the course of purification. The concentration of free aqua-ions of corresponding metals in the metal-ion buffers were calculated according to Raaflaub [2], *i.e.*, without approximations when high excess of a complexing agent is present [3]. Stability constants for citrate complexes are from Li [4].

Peptidase I was activated only by Co^{++} at the concentration of free ion $1.5 \cdot 10^{-4}$ *M* for maximal enzyme activity. Leu-β-NA, Leu-NH_2, Leu-Gly-Gly, some other tripeptides and few dipeptides were hydrolyzed. II was activated by Zn^{++}, Mn^{++} and Co^{++} at the concentrations of free ions 10^{-5}, $6 \cdot 10^{-5}$ and $6 \cdot 10^{-5}$ *M* respectively for maximal activity, the maximal values of activity for Mn^{++} and Co^{++} being approx. 60 and 35 per cent as compared with Zn^{++}. Only tripeptides were hydrolyzed by enzyme II, including those that are hydrolyzed by I. III was activated by Co^{++} and Mn^{++} to the maximal degree of activity at the concentrations of free ions $3.3 \cdot 10^{-7}$ and $1.4 \cdot 10^{-6}$ *M* respectively. The enzyme hydrolyses only a large number of dipeptides.

The other metal-ions did not show any activation of all three apoenzymes in the range of concentration

of free metal-ion 10^{-6}–10^{-3} *M* in the metal-ion buffers. The excess of activators inhibited the enzymatic reaction. The optimal pH values for I, II and III are 8.1, 7.9 and 7.3 respectively.

From the date presented, I, II and III may be identified respectively as aminopeptidase, tripeptidase and dipeptidase.

1 S. R. Himmelhoch, H. A. Sober, B. L. Vallee, E. A. Peterson and K. Fuwa, *Biochemistry, 5,* 2523 (1966).
2 J. Raaflaub, 'Methods in Biochemical Analysis', Vol. 3, pp. 301–305, Acad. Press, N.Y. - London (1956).
3 D. D. Perrin, *Crit. Revs. Anal. Chem., 5,* 85 (1975).
4 N. C. Li and J. M. White, *J. Inorg. Nucl. Chem., 16,* 131 (1960).

O14

Ascorbate Oxidase: Properties of the Copper Sites and Catalytic Activity of the Native and Type 2 Cu Depleted Enzyme

P. M. H. KRONECK, W. JAKOB, H. MERKLE

Universität Konstanz

and A. MARCHESINI

Istituto Sperimentale Per La Nutrizione Delle Piante, Turin, Italy

Ascorbate oxidase (L-ascorbate: O_2 oxidoreductase, E.C. 1.10.3.3) is the subject of numerous biological investigations. Some of the recent developments in the purification of the enzyme, the number of Cu atoms per active molecule, the stoichiometry of the different Cu sites and the mechanism of reduction by L-ascorbate have been previously reviewed [1].

Ascorbate oxidase from squash Cucurbita p.m. contains 8 Cu atoms/140 000 M_r of which 50% are detectable by electron paramagnetic resonance (EPR), *i.e.* three type-1 Cu, one type-2 Cu and four type-3 Cu [2]. The number and stoichiometry of different Cu sites depend very much on environmental conditions maintained during the process of isolation and purification, such as pH, ionic strength or presence of metal chelators. Thus, dialysis of pure enzyme against acetate buffer, pH 4.5–5.5, may lead to drastic changes in the visible region of the electronic spectra (indicated by a decrease of the purity index A_{610}/A_{330} [1]) and of the EPR spectra (indicated by a decrease of the type-1 signal). In the presence of metal chelators, such as EDTA or dimethylglyoxime [3], the type-2 center is removed selectively (= t2d enzyme) as demonstrated by EPR spectrosocpy. Depending on dialysis conditions (pH, concentration of metal chelator, aerobic *vs.* anaerobic) up to four Cu atoms will be dialysed off. As epxected, the activity of these enzyme samples is lowered to less than 4% of the original activity present.

The so-called t2d enzyme (5.8 ± 0.3 Cu/140.000 M_r) is still reduced by L-ascorbate in the absence of dioxygen, approx. 5.5 electron equivalents are used for total bleaching of the absorbance at 610 nm. Upon exposure to air, enzyme reoxidation begins as indicated by the appearance of the typical blue color (λ_{max} 610 nm) and the type-1 Cu EPR signal. By comparison with native ascorbate oxidase this seems to be a relatively slow process, complete reoxidation is achieved after a few minutes at 20 °C, pH 7.0.

The time course of the reaction of reduced ascorbate oxidase, or reduced t2d enzyme, with dioxygen was followed by stopped-flow spectrophotometry and rapid-freeze EPR spectroscopy. Prior to reaction with molecular oxygen the protein is reduced by an excess of L-ascorbate (6–8 equivalents of reductant per enzyme), hereafter mixing with O_2/buffer solution is achieved under the strict exclusion of air at temperatures between 5 and 10 °C [1]. In the first rapid phase of the reoxidation of the native enzyme (2–4 msec) the characteristic absorption spectrum and EPR signal of the L-ascorbate radical (λ_{max} 365 nm, g = 2.005) can be detected [1], due to the turnover of the enzyme in the presence of reducing substrate and dioxygen. Total reoxidation of ascorbate oxidase occurs very rapidly, within less than 8 msec at 10 °C as shown optically by the appearance of absorbance at 610 nm and by EPR. By comparison with untreated ascorbate oxidase the reduced t2d enzyme is far less reactive towards dioxygen, the reoxidation process takes several minutes under identical conditions of pH, ionic strength, temperature and oxygen concentration.

From the kinetic experiments on the anaerobic reduction of ascorbate oxidase and oxidation of the reduced enzyme conclusions are drawn concerning the modes of internal and external electron transfer. Furthermore, mechanistic aspects of the catalytic function of the multicopper oxidase are discussed.

1 P. M. H. Kroneck, F. A. Armstrong, H. Merkle and A. Marchesini, 'Ascorbic Acid: Chemistry, Metabolism and Uses', (P. A. Seib and B. M. Tolbert, eds.), *Adv. Chem. Ser., 200,* 223 (1982).
2 R. Malkin and B. G. Malmström, *Adv. Enzymol., 33,* 177 (1970).
3 L. Avigliano, A. Desideri, S. Urbanelli, B. Mondovì and A. Marchesini, *FEBS Letters, 100,* 318 (1979).