Denaturation and Reactivation of Bovine and Human Cobalt-Carbonic Anhydrases in Guanidine Hydrochloride *

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Bovine and human erythrocyte carbonic anhydrases (carbonate hydro-lyase, EC4.2.1.1) are zinc metalloenzymes which catalyze the reversible hydration of CO_2 .¹ Studies of the folding of the bovine and human Zn(II)-carbonic anhydrases in guanidine-HCl and urea have been reported in several papers²⁻¹⁰ and the presence or absence of Zn^{2+} has been shown to strongly affect the refolding kinetics.^{3,6} The zinc ion in native carbonic anhydrase can be removed and a catalytically active Co(II)-enzyme can be made.¹¹ Coordination of Co^{2+} to the active site gives rise to various absorption bands in the visible wavelength region of the spectrum.¹¹

Therefore Co^{2+} can be used as a spectroscopic probe during folding processes of the enzyme. The denaturation of bovine Co(II)-carbonic

anhydrase has been investigated, whereas attempts to renature guanidine-HCl denatured Co(II)-enzyme have so far been unsuccessful.¹² In order to elucidate the role of the metal ion in the folding of carbonic anhydrase, we have undertaken an investigation of the denaturation and reactivation of bovine and human erythrocyte Co(II)-carbonic anhydrases.

Experimental. Human carbonic anhydrase B and C were purified from human hemolysate by affinity chromatography according to Khalifah *et al.*¹³ For the preparation of bovine carbonic anhydrase C the same conditions as used for the human enzymes were applied except for the elution step. The bovine C enzyme was eluted with 0.1 M Tris- H_2SO_4 buffer, pH 7.0, containing 0.2 M NaN_3 . Protein concentrations were estimated spectrophotometrically at 280 nm assuming $A_{280\text{ nm}}^{1\%}$ values of 16.3 cm^{-1} , 18.7 cm^{-1} and 19.0 cm^{-1} for the human carbonic anhydrase B, C and bovine carbonic anhydrase C, respectively.¹⁴

The homogeneity of the preparations were analyzed by polyacrylamide gel electrophoresis¹⁵ (7.5 % acrylamide, 0.095 M Tris-glycine buffer, pH 9.5).

Bovine Co(II)-carbonic anhydrase was prepared according to Lindskog and Malmström¹⁶ and human Co(II)-carbonic anhydrase B and C were made by the method of Hunt *et al.*¹⁷

CO_2 hydration activities were measured by a colorimetric method.¹⁸ Corrections for inhibition by guanidine-HCl during the assay were performed as described earlier.⁴

Spectrophotometric measurements were made with a Perkin-Elmer Lambda 3 apparatus equipped with a 10 cm cuvette.

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Fig. 1. Effects of guanidine-HCl on the CO_2 hydration activity and absorbance at 550 nm of Co(II)-carbonic anhydrases. Fractional loss of enzyme activity of bovine Co(II)-carbonic anhydrase C, ($\square$); human Co(II)-carbonic anhydrase B, ($\circ$); human Co(II)-carbonic anhydrase C, ($\bullet$). Fractional change in absorbance at 550 nm for human Co(II)-carbonic anhydrase B, ($\star$). Protein concentrations 0.1 mg/ml in 0.1 M Tris- H_2SO_4 buffer, pH 7.5; temp. 23 °C.

Fig. 2. Time courses for the reactivation of Co(II)-carbonic anhydrases denatured in 5.0 M guanidine-HCl. Final concentrations of protein and guanidine-HCl during reactivation: Bovine Co(II)-carbonic anhydrase C (0.25 mg/ml, 0.5 M), (□); human Co(II)-carbonic anhydrase B (0.025 mg/ml, 0.5 M), (○); human Co(II)-carbonic anhydrase C (0.025 mg/ml, 0.3 M), (●). Buffer: 0.1 M Tris-H₂SO₄, pH 7.5; temp. 23 °C.

Guanidine-HCl was prepared from guanidinium carbonate¹⁹ and recrystallized and the concentration was determined refractometrically.²⁰

Results. The stabilities of the bovine and human Co(II)-carbonic anhydrases in guanidine-HCl were investigated by incubation of the enzymes for 2 h in various concentrations of the denaturing agent. After this exposure to the denaturant the CO₂ hydration activity was measured and the results are illustrated in Fig. 1. The

denaturation of the Co(II)-enzymes is a highly cooperative process like that of the Zn(II)-enzymes. The midpoints of the transition profiles are reached at guanidine-HCl concentrations of 1.0, 1.2 and 1.5 M for the human Co(II)-carbonic anhydrase C, B and bovine Co(II)-carbonic anhydrase C, respectively. Corresponding curves for the Zn(II)-enzymes were recorded in parallel for comparative purposes and the following midpoints of denaturation were obtained: 1.0, 1.4 and 1.6 M for the human C, B and bovine C enzyme, respectively. The main absorbance band resulting from the Co²⁺ substitution of Zn²⁺ in the active site of the enzyme has a maximum at 550 nm. For human Co(II)-carbonic anhydrase B the change in absorbance at this wavelength was also monitored as a function of the guanidine-HCl concentration. As shown in Fig. 1 this absorbance decrease coincides with the inactivation curve within experimental error.

The time courses for the reactivation of the bovine and human Co(II)-carbonic anhydrases are shown in Fig. 2. In the reactivation experiments Co(II)-enzymes were first denatured in 5.0

M guanidine-HCl for 2–3 h. The enzymes were then reactivated by rapid dilution of the denaturant with buffer. Optimal reactivation of the human Co(II)-carbonic anhydrases was obtained when the Co(II)-enzymes B and C were each diluted to a protein concentration of 0.025 mg/ml and a concentration of guanidine-HCl of 0.5 M and 0.3 M, respectively. After 24 h of reactivation the human Co(II)-carbonic anhydrases B and C recovered about 80 and 90 %, respectively, of the specific CO₂ hydration activities of the native Co(II)-enzymes. Increased protein concentrations during the reactivation process gave rise to decreasing yields of active enzymes. For the bovine Co(II)-carbonic anhydrase C, a reactivation of 95 % could be obtained in 2 h (Fig. 2) after dilution to 0.5 M guanidine-HCl and a 10-fold higher protein concentration than for the human enzymes. The half-times of reaching the final values of reactivation were 13, 15 and 23 min for the bovine Co(II)-carbonic anhydrase C, human Co(II)-carbonic anhydrases C and B, respectively, (Fig. 2). The corresponding half-times for the Zn(II)-enzymes under identical reactivation conditions were 6, 9 and 4 min.

Discussion. The results of this study indicate that the stability of the tertiary structure of carbonic anhydrase with regards to denaturation in guanidine-HCl is dependant on the metal ion. Substitution of Zn²⁺ in the active site by Co²⁺ seems to some extent lower this stability (Fig. 1). For the bovine Co(II)-carbonic anhydrase C a similar trend has been reported, when the denaturation was followed by various physical parameters.¹² Furthermore, the bovine apoenzyme C has been shown to be considerably less

stable than the holoenzyme.^{3,6} The concomitant drop of the visible absorbance and activity with increasing concentrations of guanidine-HCl for the human Co(II)-carbonic anhydrase B (Fig. 1) suggests that the inactivation of the enzyme is accompanied by dissociation of Co^{2+} or a markedly different coordination of the Co^{2+} in an inactive state of the enzyme. Conclusions regarding the existence of different Co^{2+} -intermediates on the folding pathway must wait until kinetic data are available.

The main result of this work is that conditions have been found for practically full reactivation of bovine and human Co(II)-carbonic anhydrase C. For the corresponding human B enzyme, a somewhat lower yield (80 %) of active enzyme was recovered. The reported difficulties in achieving renaturation of the bovine Co(II)-carbonic anhydrase C are probably due to the use of dialysis to remove the denaturant.¹² This relatively slow procedure might favour the formation of "incorrectly" folded states, which have been shown to accumulate in the transition region of the denaturation of human Zn(II)-carbonic anhydrase B.⁴ The existence of similar states has also been discussed for the bovine Zn(II)-carbonic anhydrase C.¹⁰ By rapid dilution well below the midpoints of the transition curves, the formation of such dead-end conformations can, however, be minimized.

Since protein concentrations of the order of 0.25 mg/ml can be used for the reactivation of bovine Co(II)-carbonic anhydrase C, it should be possible to employ the Co^{2+} as a spectroscopic probe in kinetic studies of the role of the metal ion during the refolding of this enzyme.

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1. Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O. and Strandberg, B. In Boyer, P. D., Ed., *The Enzymes*, Academic, New York 1971, Vol. 5, p. 587.
2. Edsall, J. T., Mehta, S., Myers, D. V. and Armstrong, J. McD. *Biochem. Z.* 345 (1966) 9.
3. Yazgan, A. and Henkens, R. W. *Biochemistry* 11 (1972) 1314.
4. Carlsson, U., Henderson, L. E. and Lindskog, S. *Biochim. Biophys. Acta* 310 (1973) 376.
5. Wong, K.-P. and Tanford, C. J. *Biol. Chem.* 248 (1973) 8518.
6. Wong, K.-P. and Hamlin, L. M. *Arch. Biochem. Biophys.* 170 (1975) 12.
7. Carlsson, U., Aasa, R., Henderson, L. E., Jonsson, B.-H. and Lindskog, S. *Eur. J. Biochem.* 52 (1975) 25.
8. Ko, B. P. N., Yazgan, A., Yeagle, P. L., Lottich, S. C. and Henkens, R. W. *Biochemistry* 16 (1977) 1720.
9. Stein, P. J. and Henkens, R. W. *J. Biol. Chem.* 253 (1978) 8016.
10. McCoy, L. F., Rowe, E. S. and Wong, K.-P. *Biochemistry* 19 (1980) 4738.
11. Lindskog, S. *J. Biol. Chem.* 238 (1963) 945.
12. McCoy, L. F. and Wong, K.-P. *Biopolymers* 18 (1979) 2893.
13. Khalifah, R. G., Strader, D. J., Bryant, S. H. and Gibson, S. M. *Biochemistry* 16 (1977) 2241.
14. Nyman, P. O. and Lindskog, S. *Biochim. Biophys. Acta* 85 (1964) 141.
15. Smith, I. *Chromatographic and Electrophoretic Techniques*, 2nd Ed., Heinemann, London 1968, Vol. 2, p. 365.
16. Lindskog, S. and Malmström, B. G. *J. Biol. Chem.* 237 (1962) 1129.
17. Hunt, J. B., Rhee, M.-J. and Storm, C. B. *Anal. Biochem.* 79 (1977) 614.
18. Rickli, E. E., Ghazanfar, S. A. S., Gibbons, B. H. and Edsall, J. T. *J. Biol. Chem.* 239 (1964) 1065.
19. Nozaki, Y. *Methods Enzymol.* 26 (1972) 43.
20. Kielley, W. W. and Harrington, W. F. *Biochim. Biophys. Acta* 41 (1960) 414.

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