Vol. 148, No. 3, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 13, 1987 Pages 1196-1201

# THE DISSOCIATION OF CARBON MONOXIDE FROM THE ALPHA AND THE BETA SUBUNITS OF HUMAN CARBONMONOXY HEMOGLOBIN

Michele Samaja[a] and Ermanna Rovida[b]

[a,b]Dipartimento di Scienze e Tecnologie Biomediche, Universita' di Milano, and Centro Studi di Fisiologia del Lavoro Muscolare, Consiglio Nazionale delle Ricerche, Istituto Scientifico San Raffaele, 20132 Milano, ITALY

Received September 8, 1987

We have measured the intrinsic CO dissociation rates from the subunits of the human hemoglobin tetramers $(\alpha CO\ \beta NO)_2$ and $(\alpha NO\ \beta CO)_2$ using microperoxidase and a stopped-flow spectrophotometer. The dissociation of NO is negligible. The rate constants for the $\alpha$ and the $\beta$ subunits are similar ($0.014\ s^{-1}$ vs. $0.011\ s^{-1}$, respectively, at pH 7, 20 C; and $0.016\ s^{-1}$ for both in the presence of inositol hexaphosphate), indicating that they are equivalent in the first step of the CO dissociation. Therefore, the chain unequality observed in the third and fourth steps (Samaja, M., Rovida, E., Niggeler, M., Perrella, M., and Rossi-Bernardi, L. (1987). J. Biol. Chem.: 262, 4528-4533) are not due to the intrinsic properties of the subunits, but to the conformational state of the molecule. 

Human hemoglobin may exist in ten different forms during the reaction with ligands, because of its assembly with two $\alpha$ and two $\beta$ subunits, and the presence of a symmetry plan between the dimers. We have recently reported that the two subunits dissociate CO at different rates in the intermediates with 1 and 2 ligands [1], but since this feature was observed in the correspondence of a conformational switch of the molecule, it was difficult to assign this heterogeneity to different intrinsic properties of the chains or to the different conformational states of the intermediates. Previous work concerning the ligand dissociation from the isolated chains [2], oxyhemoglobin [3], and $Hb(CO)_4$, [4-7], was unable to distinguish the intrinsic functional properties of the subunits within the tetramer.

The abbreviations used are: Hb, deoxy (unliganded) hemoglobin; $Hb(CO)_4$, carboxyhemoglobin; $Hb(NO)_4$, nitrosyl hemoglobin; IEF, isoelectric focusing; IHP, inositol hexaphosphate; MP, microperoxidase. The notation (L1/L2), where L1 and L2 represent two different ligands, means that the chains of one genotype are bound to the ligand L1, and those of the other genotype are bound to the ligand L2.

0006-291X/87 $1.50

The aim of this work is to determine the kinetic properties of the subunits in the fully liganded tetramer. We have obtained the hemoglobin species with the chains of one genotype ($\alpha$ or $\beta$) bound to CO, and those of the other genotype bound to NO. These species are then reacted with microperoxidase (MP) under pseudomonomolecular conditions with the rate limiting step represented by the dissociation of the ligand [1]. Since the dissociation of NO is 1000 times slower than that of CO, the observed initial rates represent the dissociation of CO from one of the subunits of the fully liganded tetramer. We will show that the chains of human hemoglobin are virtually similar in the first step of the dissociation of CO, both in the presence and in the absence of inositol hexaphosphate (IHP), and we will discuss the implications of this observation.

## Materials and Methods

Reagents. We have used a 20 mM potassium phosphate / 50 mM KCl / 1 mM EDTA buffer (pH 7 at 25 C), equilibrated with gaseous CO or NO by stirring in closed vessels for 30 min at 20 C. The gas solubility (1.0 and 1.9 mM, respectively) was measured from the stoichiometry of the binding to known amounts of deoxygenated hemoglobin. Sodium dithionite (Carlo Erba, Italy) and microperoxidase (Sigma Co., MO) were obtained as previously explained [1].

Hemoglobin. The valence intermediates (+/O2) were obtained from half-oxidized hemoglobin at equilibrium with $O_2$ and potassium exacyanoferrate(III)(2 min incubation at 0 C), by chromatography with CM52 [8]. The protein concentration was measured by the Drabkin's method, and is expressed on a molar heme basis. The extinction coefficients used at 590 nm for MPCO, MPNO, $MP_4$, $Hb(CO)_4$, $Hb(NO)_4$ and Hb, are 1.36, 2.51, 0.88, 2.5, 6.75 and 6.51 $mM^{-1}$ $cm^{-1}$, respectively.

The (CO/NO) species were obtained carrying out the following operations at 0 C. Load half milliliter of 1 mM valence intermediate (+/O2) in a tonometer, and add 0.8 mM KCN to mask the oxidized hemes as CN-met hemes. Flush with $N_2$ at 300 ml/min for 30 min and check the complete removal of $O_2$ by a polarographic method [9]. Add 0.5 ml of NO-buffer and remove excess NO by further 5 min tonometry. Purge a 1 ml gas-tight syringe (Hamilton-Bonaduz, Switzerland) with CO-buffer containing 7 mg/ml dithionite, and leave 0.25 ml of buffer in the syringe. Withdraw anaerobically 0.75 ml of the hemoglobin solution by the same syringe, and gently mix the content 1 or 2 min by rotation while warming to room temperature. By this way, the CO in the buffer, that is equimolar to the CN-met hemes, binds to the deoxygenated hemes that are formed in the reaction of oxidized hemes with dithionite.

Data collection and analysis. The hemoglobin solution was immediately reacted at 20 C with 0.6 mM MP and 140 mg/ml dithionite in a Durrum-Gibson stopped-flow apparatus (Dionex, Palo Alto, CA), equipped with a 2-cm path length cuvette, and with a reagents mixing ratio of 1:3.8. The kinetics was followed at 590 nm, the data were collected by a Nicolet 3091 digital oscilloscope (Nicolet Instruments Corp., Madison, MA), and tranemitted to a PC-350 computer (Digital Equipment Corp., Maynard, MA). A standard first-order treatment of the data was used to calculate the values of the rate constants.

## Results

The purity of the valence intermediates (+/O2) produced by ion-exchange chromatography is routinely better than 98%, as checked by IEF at -25 C [1]. Because of the impossibility to perform any such control on the final (CO/NO) product (all the four-liganded species have the same pI and thus they are not resolved in an electric field), we have tested separately the various steps of the protocol to obtain the (CO/NO) species using appropriate solutions of pure hemoglobin. These tests (not shown) yielded a virtually complete recovery of the final product. The absorbance change for the dissociation of CO from two hemes of hemoglobin and its binding to MP with no rearrangement of NO is maximal at 590 nm (0.240 units), as calculated from the spectra of the pure components. The observed absorbance change at the apparent end of the reaction (about 200 s) is 0.225 units, and thus slightly smaller than predicted. The dissociation of NO from hemoglobin and its binding to MP should produce an additional change of 0.070 units.

Figure 1 shows the first order plot of the CO dissociation from the species $(\alpha CO\ \beta NO)_2$ and $(\alpha NO\ \beta CO)_2$, at pH 7, 20 C, without and with added IHP ([IHP]/[$Hb_4$]=2). The intrinsic rate constants, calculated from the first-order plot in the 0 to 15 s range, are 0.014 $s^{-1}$ and 0.011 $s^{-1}$, for the and the chains, respectively. In the presence of IHP, both values increased to 0.016 $s^{-1}$. The kinetics of the CO dissociation from $Hb(CO)_4$, and from the species $(\alpha CO\ \beta)_2$ and $(\alpha\ \beta CO)_2$ [1] are added in the figure for comparison.

## Discussion

The main finding of this work is the subunit equivalence in the first step of the CO dissociation from $Hb(CO)_4$. This is in contrast with previous findings on the isolated chains that the CO dissociation from the $\alpha$ chains is twice faster than that from the $\beta$ chains [2]. The loss of the pseudo-monomolecular character of our kinetics after 40 s, likely due to ligand and dimer rearrangements, made it difficult to perform any investigation on the second step of the CO dissociation.

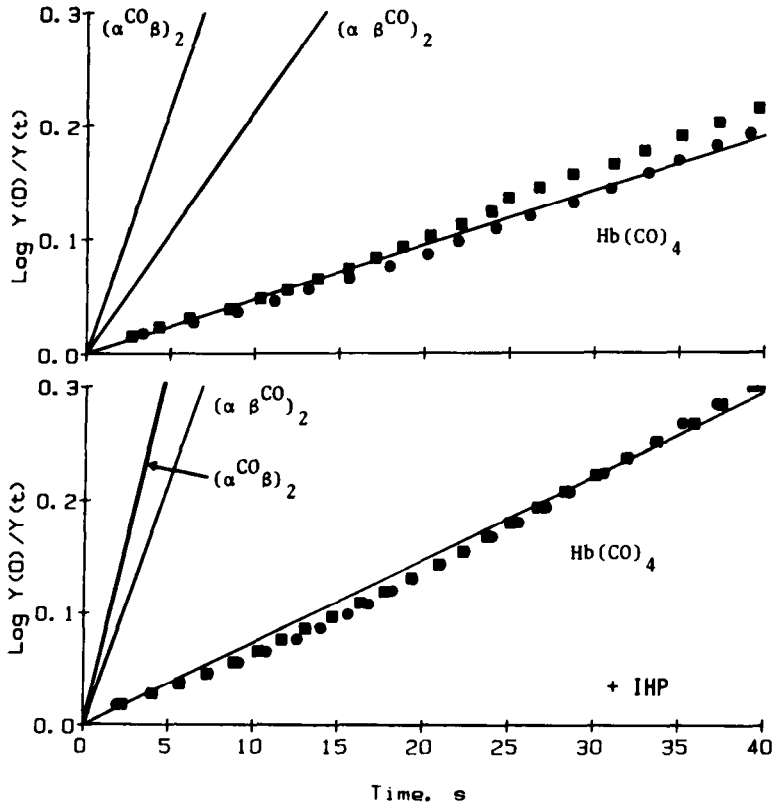


Figure 1. First order plot (Y=fractional CO saturation) of the CO dissociation from $(\alpha NO\ \beta CO)_2$ ( ● ) and $(\alpha CO\ \beta NO)_2$ ( ■ ), in the absence (upper panel) and in the presence (lower panel) of IHP. The kinetics are obtained at [heme]=0.1 mM (after mixing), pH 7, 20 C. The lines represent the CO dissociation (from left to right) from $(\alpha CO\ \beta)_2$, $(\alpha\ \beta CO)_2$, and $Hb(CO)_4$, as in ref.1, under the same conditions. The values of the rate constants are 0.10, 0.049, 0.011, and 0.15, 0.10, 0.017 $s^{-1}$, for $(\alpha CO\ \beta)_2$, $(\alpha\ \beta CO)_2$, and $Hb(CO)_4$, in the absence and in the presence of IHP, respectively.

The possibility that the presence of two NO-hemes affects the reactivity of the CO-hemes in the same molecule is ruled out by the observation that the kinetics of the CO dissociation in the presence of IHP is virtually similar to that measured in its absence. In facts, IHP induces a conformational change in $Hb(NO)_4$ [6], but not in $Hb(CO)_4$ [1], therefore the lack of effect of IHP on the CO dissociation rates implies that no NO-mediated changes are present.

Also, NO does not dissociate appreciably during the kinetics, and therefore the observed absorbance change is due to the dissociation of CO only, because: a) the dissociation of NO from $Hb(NO)_4$ ($k=1x10^{-5}\ s^{-1}$ [2]) is three ord-

ers of magnitude slower than the dissociation of CO from $Hb(CO)_4$ ($k=0.011\ s^{-1}$) under similar, although not identical conditions; b) the observed absorbance change at the end of the reaction is consistent with the dissociation of CO only from the (CO/NO) species.

Finally, the free dimers that are present at the start of the reaction do not interfere with the measured rate constants, even if their amount is 7% of total protein, as calculated from the reported tetramer to dimer dissociation constant ($k=1x10^{-6}$ M, [10]), and the heme concentration. Free dimers can affect the kinetics of CO dissociation in two ways: a) they may dissociate CO slower than the tetramers because of the lack of cooperativity, but the rate constants were measured in the initial portion, where the CO dissociation from the tetramer is rate-limiting; b) they may rearrange before the reaction altering the starting distribution of the hemoglobin species, but this is possible only for hemoglobin solutions containing relevant amounts of asymmetrical species, that seems unlikely from the control experiments that yielded an almost complete recovery of symmetrical species. No rearrangement of the ligands is possible when obtaining the (CO/NO) species because all the hemoglobin species that are produced have no free sites for ligand binding. Ligand rearrangement during the reaction is prevented by the presence of excess MP.

We can thus conclude that the first step of the dissociation of CO from $Hb(CO)_4$ is independent of the subunit, in contrast to the third and fourth steps, where the $\alpha$ chains dissociate CO twice faster than the $\beta$ chains [1]. This finding indicates that the functional differences between the $\alpha$ and the $\beta$ chains in the intermediates with 1 and 2 ligands are due to different quaternary conformations of such intermediates rather than to different reactivities of the subunits.

Acknowledgements

This work was supported by grant no. 86.00139 by the Consiglio Nazionale delle Ricerche, Roma.

## References

1. Samaja, M., Rovida, E., Niggeler, M., Perrella, M., and Rossi-Bernardi, L. (1987). J. Biol. Chem. 262, 4528-4533
2. Moore, E.G., and Gibson, Q.H. (1976) J. Biol. Chem. 251, 2788-2794
3. Cordone, L., Cupane, A., and Fornili, S.L. (1983) Biopolymers, 22, 1677-1696
4. DeYoung, A., Pennelly, R.R., Tan-Wilson, A.L., Noble, R.W. (1976) J. Biol. Chem. 251, 6692-6698
5. Antonini, E., and Gibson, Q.H. (1960) Biochem. J. 76, 534-536
6. Salhany, J. M., Ogawa, S. and Shulman, R. G. (1974). Proc. Nat. Acad. Sci. U.S.A. 71, 3359-3362
7. Sharma, V. S., Schmidt, M. R. and Ranney, H. M. (1976). J. Biol. Chem. 251, 4267-4272
8. Tomoda, A. and Yoneyama, Y. (1981). Anal. Biochem. 110, 431-436
9. Samaja, M., and Rovida, E. (1983). J. Biochem. Biophys. Methods 7, 143-152
10. Barksdale, A. D., and Rosenberg, A. (1978). J. Biol. Chem. 253, 4881-4885