Bioenergetics of the Heart at High Altitude: Environmental Hypoxia Imposes Profound Transformations on the Myocardial Process of ATP Synthesis

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The low concentration of O_2 in the thin air at high altitude is undoubtedly the reason for the remarkable modifications in the structure and function of the heart, lung, and blood of humans permanently living under these conditions. The effect of natural hypoxia on the energy metabolism of the cell is however not well understood. Here we study the proces of ATP synthesis in the heart of guinea pigs native to high altitude (4500 m) as compared with those native to sea level. The following are the novel findings of this study. (1) The *rates and extents of ATP synthesis* in the presence of low concentrations of ADP ($<30\ \mu$M) are significantly higher at high altitude than at sea level. (2) The Hill coefficient, i.e. the *degree of cooperativity* between the three catalytic sites of the ATP synthase, is lower at high altitude ($n = 1.36$) than at sea level ($n = 1.94$). (3) Both, the *affinity for ADP* and the *fractional occupancy* of the catalytic sites by ATP, are higher at high altitude than at sea level but the P_{50}, i.e. the concentration of ADP at which 50% of the catalytic sites are filled with ADP and/or ATP, is the same ($\sim$74.7 μM). (4) In the physiological range of ADP concentrations, the phosphorylation potential ΔG_P is significantly higher at high altitude than at sea level. It is concluded that the molecular mechanism of energy transduction is profoundly modified at high altitude in order to readily and efficiently generate ATP in the presence of low concentrations of O_2 and ADP.

KEY WORDS: Oxygen; hypoxia; mitochondria; oxidative phosphorylation; ATP synthesis.

INTRODUCTION

The remarkable ability of native humans and animals to cope with the hypoxic environment of high altitude (HA) has for centuries intrigued biologists and physiologists. It is indeed amazing to see native young people playing soccer for hours at altitudes of 4500 m when newcomers from sea level (SL) can barely walk for a few minutes (personal experience). The increments in hemoglobin as well as in the volume of the chest and in the thickness of the right ventricle of the heart of people permanently living at high altitudes are undoubtedly the consequence of the hypoxic environment (Reynafarje, 1962, 1966). The modifications observed in the hematologic, respiratory, and cardiovascular systemes are however not necessarily proportional to the degree of adaption to these environments. Thus, the fittest individuals among healthy young people permanently living at HA are those having the smallest increments in the number of red blood cells, the volume of the lungs, and the thickness of the right ventricle (Velasquez and Reynafarje, 1966). It thus seems that during the process of adaptation to high altitude it may be more important to improve the transformation of the electron transfer potential of O_2 reduction into the phosphoryl transfer potential of ATP than to improve the transport of O_2 to the tissues. We show here that precisely in range of ADP concentrations considered normal for the resting muscle, the heart of guinea pigs adapted to hypoxic environments are able to synthesize ATP and generate ΔG_P with high efficiency. In general these results show that the

Key to abbreviations: SMP, submitochondrial particles; HA, high altitude; SL, sea level; ΔG_P, phosphorylation potential.

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process of adaptation to HA involves profound alterations in the structure and function of the ATP synthase so that ADP is bound with higher affinity and ATP is released with a lower degree of catalytic cooperativity.

EXPERIMENTAL PROCEDURES

Source of Enzymes, Chemicals, and Materials

Villagers permanently living in the Andes of South America at altitudes of near 4500 m as well as those living at SL raise guinea pigs as a source of meat. The heart of six guinea pigs from HA and six from SL were excised in their place of origin under aseptic conditions, placed in dry ice, and personally transported to USA to be analyzed within 2 days. The standard reaction mixture (in 1.0 mL of final volume at pH 7.05 and 24°C) consisted of 200 mM of sucrose, 50mM of KCl, 10mM of K-NaPi, pH 7.05, and 2 mM of $MgSO_4$. The O_2 concentration of the air-saturated medium at SL was 230 μM as experimentally determined (Reynafarje *et al.*, 1985). ATP, ADP, horse-heart cytochrome *c* (Type IV), NADH, succinate, and fumarate were products of Sigma Chemical Co. The 1243-200 monitoring reagent for ATP, a mixture of luciferin and luciferase, was a product of Bio Orbit. All other reagents were of analytical grade purity. The luminometer used to monitor ATP synthesis was a product of LKB (Wallac Model 125). The oxygen electrode, constructed and used as previously described, had a 90% response time of about of 10 ms (Reynafarje and Davies, 1990). The reaction chamber was fitted with the oxygen electrode and a combination pH electrode (Beckman Altex 531167). The electrical signals coming from the O_2 electrode and the luminometer were fed into a KIPP & ZONEN multichannel recorder usually running at a chart speed of 120 cm/min.

Chemiluminescent Method to Determine the Extent and Rates of ATP Synthesis

The method used to determine the synthesis of ATP in the presence of high concentrations of O_2 was similar to that employed by Lemasters and Hackenbrock (1997). However, in order to determine simultaneously the processes of ATP synthesis and O_2 consumption in homogenates of whole tissues, we used the following procedure. A piece of the right ventricle was finely minced over ice, dispersed in the standard reaction medium at a concentration of 125 mg of tissue per mL, and gently homogenized by hand with a Ten-Broeck homogenizer untill all visible particles disappear. Unless otherwise indicated,

60 μL of the homogenate was mixed in the barrel of a closed Hamilton syringe with 40 μL of the standard medium containing 10mM of each fumarate and succinate. The mixture was incubated for at least 20 min to consume all the O_2 and to fully reduce the mitochondrial membrane under anaerobic conditions. Reactions were initiated by rapidly injecting the *anaerobic* content of the Hamilton syringe into 0.9 mL of medium containing ~200 μM O_2, 50 μL of a dilution of the ATP-monitoring reagent in 5 mL of distilled water, and different concentrations of ADP. The electrical signals coming from the luminometer induced by the change in ATP and O_2 concentration were suitably amplified up to 10,000 times by changing the current (10^{-6}–10^{-8} A) and/or the voltage (1 mV to 10 V) of the recorder. A magnetic bar rotating at speeds of ~1000 rpm served to continuously mix the contents of the cell. The extent and initial rates of O_2 consumption were simultaneously recorded only in reactions initiated by adding very low concentrations of O_2 like in those depicted in Fig. 1. Changes in O_2 concentration in reactions containing more than 200 μM O_2 (air-saturating mediums) were not recorded because the amount of O_2 consumed at the time the net synthesis of ATP ceased was relatively insignificant.

To evaluate the extent of ATP synthesis we used a standard curve constructed in the presence of heat-denatured tissue by plotting the intensity of light emission versus various concentrations of ATP (10^{-6}–10^{-12} M) as
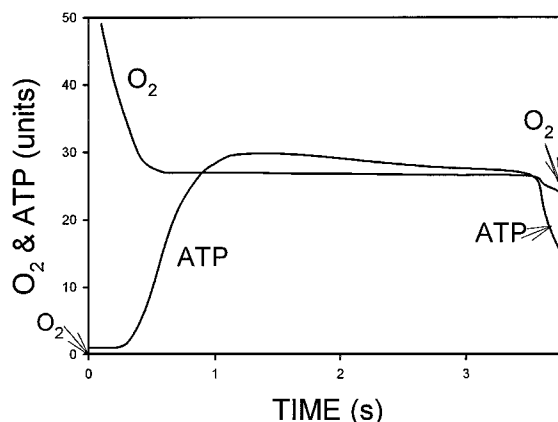

Fig. 1. Simultaneous and continuous determinations of ATP synthesis and O_2 consumption in reactions catalyzed by heart homogenates. The standard reaction mixture (see Experimental Procedures) contained 5.0 mg (w/w) of the HA heart homogenate supplemented with 250 μM of ADP and 10.0 mM of succinate. The reaction was initiated at zero time (left arrow) by injecting 6.9 nmol of O_2 into an anaerobic suspension of the homogenate and the changes in ATP and O_2 concentration followed for 3.7 s, after which the time scale in the abscissa was changed to minutes. One unit in the ordinate represents 0.276 nmol of O and 0.105 nmol of ATP.
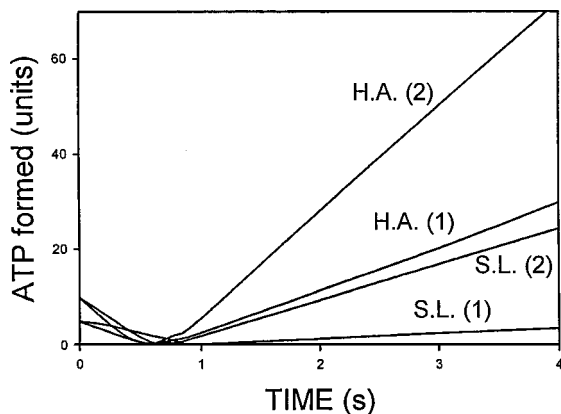
Fig. 2. Effect of ADP concentration on the initial rates of ATP synthesis at sea level and high altitudes. Reactions were initiated by injecting 8.3 mg (w/w) of anaerobic and fully reduced homogenates of the right ventricle (see Experimental Procedures) into reaction mixtures containing 10.0 mM of succinate, 230 μM of O_2, and either 1.0 or 2.0 μM of ADP (figures in parentheses). One unit in the ordinate is equivalent to 0.209 pmol of ATP. The traces of O_2 consumption are not shown because the fraction of O_2 consumed is so small that changes in O_2 concentration are not apparent.

previously described (Reynafarje and Pedersen, 1996). The initial rates of ATP synthesis were determined at steay state by measuring the steepest slope of the traces (see Fig. 2). The phosphorylation potential ΔG_P was calculated using a modification of the generally employed mass action ratio ($\Gamma = $ [ATP]/[ADP] [Pi]). Considering that the free energy (ΔG) of a reaction is equal to the difference in the free energy content of its reactants ($\Delta G = $ RT ln[the actual product/substrate rations] $-$ RT ln[the product/substrate ratios at equilibrium]), we used the following equation:

$$\Delta G_P = \text{RT} \ln [\text{ATP}] [\text{S}] [\text{H}_2\text{O}]/[\text{ADP}] [\text{Pi}] [\text{O}_2]^{1/2} [\text{SH}_2]$$
$$- \text{RT} \ln \text{K}_{eq} \qquad (1)$$

where RT lnK_{eq}, the standard free energy change of ATP hydrolysis at equilibrium, was considered to be -7.3 kcal/mole. S and SH_2 represent respectively the oxidized and reduced forms of the respiratory substrate (10 mM of each fumarate and succinate). However, since under current conditions the fraction of SH_2 (succinate) consumed when the net synthesis of ATP ceased was negligible, the S/SH_2 ratio was considered to be 1.0 and Eq. (1) was reduced to the mass action ratio except for the inclusion of the initial concentration of O_2 (200 μM).

To evaluate the degree of cooperativity in the process of ATP synthesis, we used the Hill equation as applied for the following equilibrium:

$$\log (Y/1 - Y) = n \log [\text{ADP}] - n \log P_{50} \qquad (2)$$

where Y represents the fraction of the catalytic sites that are occupied by ADP (and consequently by ATP) at the time net synthesis of ATP ceases and equilibrium between synthesis and hydrolysis is attained. The value of Y can range from 0 (all catalytic sites empty) in the absence of ADP to 1.0 (all catalytic sites filled) in the presence of saturating concentrations of ADP. The unoccupied sites $(1 - Y)$ are equal to the difference between the amount of ATP formed in the presence of maximal concentrations of ADP (all catalytic sites occupied) and the amount of ATP formed (Y) at every initial concentration of ADP.

The Hill coefficient (n) is the slope of the line that results from plotting $\log (Y/1 - Y)$ versus $\log$ [ADP]. The value of n increases with the degree of cooperativity to a maximum that is equal to the number of catalytic binding sites in the synthase. P_{50} represents the concentration of ADP at which 50% of catalytic sites are filled with ATP, i.e when $Y = 0.5$.

Validation of the Method to Determine the Synthesis of ATP in Reactions Catalyzed by Homogenates of Whole Tissues

The entire array of ATPases, phosphatases, and adenylate kinases present in a homogenate of whole tissue constitutes a serious potential pitfall to determine the exclusive synthesis of ATP by the ATP synthase. However, the synthase is the only enzyme that, even in the presence of high concentrations of ADP, cannot catalyze the *endergonic* synthesis of ATP in the absence of O_2. Thus, although the method detects extremely low concentrations of ATP ($<10^{-18}$ M), Fig. 1 shows that even in the presence of 250 μM of ADP, an anaerobic homogenate of the whole tissue (5.0 mg w/w) does not start synthesizing ATP until a small amount of O_2 (6.9 μM) is added. Furthermore, because the hydrolytic process cannot begin until ATP appears in the medium, the activity of any contaminating myokinase or ATPase will be initially negligible. In fact, Fig. 1 shows that while the synthesis of ATP and the consumption of O_2 proceed at the respective rates of 79 and 86.5 nmol per min per mg of *whole tissue* (*w/w*), the *net hydrolysis* of ATP takes place at almost negligible rates (see ATP and O_2 traces after 1 s of reaction). In reality, the kinetics and thermodynamics of ATP synthesis catalyzed by homogenates of whole tissue are no different than those catalyzed by intact *mitochondria or submitochondrial particles* in the presence of high concentrations of O_2 (Lemasters and Hackenbrock, 1997; Matsuno-Yagi and Hatefi, 1990; Perez and Ferguson, 1990; and personal observations). Finally, the validity of this new and simple method is not at all impaired by the actual presence

of potentially spurious reactions, because the study *compares* two samples (guinea pigs from SL and HA) assayed under identical conditions.

RESULTS AND DISCUSSION

The Initial Rates of ATP Synthesis Are Significantly Higher at High Altitudes Than at Sea Level

Consistent with the textbook statement that "the most important factor in determining the rate of oxidative phosphorylation is the level of ADP" (Stryer, 1995), the results depicted in Fig. 2 show that the rates of ATP synthesis are extremely sensitive to ADP concentration. It is therefore remarkable that even at very low concentrations of ADP (1.0 and 2.0 μM) the *initial rates* of ATP synthesis are many times faster at HA than at SL. Note, however, that when the concentration of ADP is 1.0 μM, the rates of synthesis are more than 7.7 times faster at HA than at SL, whereas when the ADP concentration is twice as much (2.0 μM) the rates of synthesis are only 2.7 higher at HA than at SL. This lack of direct correlation between ADP concentration and ATP synthesis is most likely due to the fact that the synthesis of ATP takes place through a cooperative process in which the catalytic sites of the synthase bind ADP with *higher affinity* at HA than at SL (see below).

The Hypoxic Environment of High Altitude Increases the Affinity of the ATP Synthase for ADP

The results depicted in Fig. 3 provide direct experimental evidence that the thermodynamic correlation between *net synthesis* of ATP and ADP concentration is sigmoidal. The relevance of this finding to the mechanism of mitochondrial energy transduction is evident. First, past reports emphasize that the mitochondrial process of ATP synthesis follows a hyperbolic time course obeying a Michaelis–Menten mechanism in which the kinetics of ATP synthesis at *low concentrations of ADP* are essentially of *first order* (LaNoue and Doumen, 1995). Second, other reports indicate that in well-coupled mitochondria the thermodynamic correlation between ATP synthesis and ADP concentration is linear, justifying the concept that the ADP/O ratio is equivalent to the ATP/O stochiometry (Chance and Williams, 1955). Importantly, Fig. 3 shows that the correlation between ATP synthesis and ADP concentration is not hyperbolic but sigmoidal and that at ADP concentrations lower than 30.0 μM the synthesis of ATP is higher at HA than at SL. In
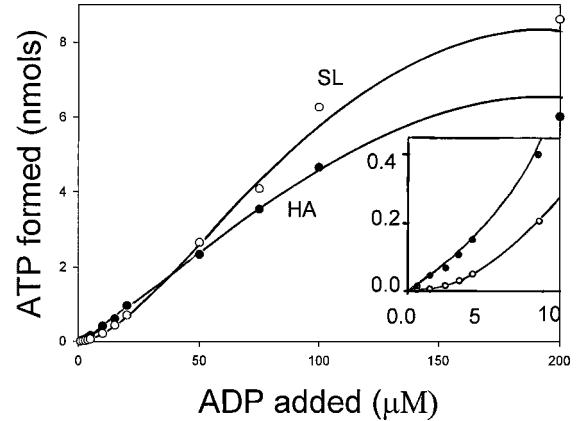

Fig. 3. Effect of ADP concentration on the sigmoidal synthesis of ATP at sea level and high altitude. The experimental conditions are as described for Fig. 2. Reactions were initiated by injecting 8.3 mg (w/w) of homogenates of right ventricles into reaction cells containing the concentrations of ADP indicated in the abscissa. The extents of ATP formed, calculated the moment the net synthesis ceases (see Fig. 1), represent the average of at least two determinations in each of the two samples. The inset is an expansion of Fig. 3 in the region of ADP concentrations between 1 and 12 μM.

fact, the inset in Fig. 3 shows that at any concentration of ADP lower than 12 μM there are no overlapping of values in at least 30 experiments in each group of animals ($p < 0.01$). Figure 4 shows that at the physiological concentrations of ADP that in all probability exist in the cell, the *initial rates of ATP synthesis* are also significantly higher at HA than at SL. It is therefore inferred that the hypoxic environment of HA induces a profound structural and functional modification of the
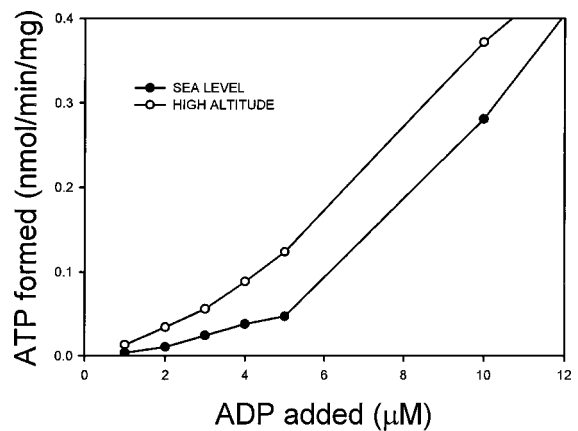

Fig. 4. Initial rates of ATP synthesis in the presence of low concentrations of ADP. Assay conditions are exactly as described in Fig. 3. The difference in the *initial rates* of ATP synthesis at HA and SL is statistically significant ($p < 0.01$).
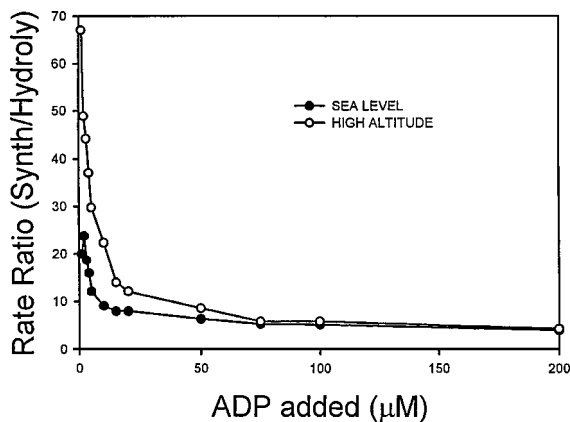
Fig. 5. Ratio between initial rates of ATP synthesis and ATP hydrolysis in the heart of guinea pigs native to sea level and high altitude. Assay conditions as described in the legend of Fig. 2, except that the amount of tissue homogenate varied between 3 and 7 mg (w/w). The *initial rates* of ATP were measured during the first 3 s of the reaction (see Fig. 2) and the *rates of net* hydrolysis immediately after the cessation of the net syntheses of ATP (see ATP trace in Fig. 1). Values represent average of duplicate experiments.

Fig. 6. Effect of ADP concentration on the Hill coefficient and the fractional occupancy of the ATP synthase catalytic sites at sea level and high altitudes. Figure 6 was constructed with date presented in Fig. 3. The lines were fitted by linear regression analysis. The Hill coefficient n and P_{50} were calculated as indicated under Experimental Procedures. Values represent average of at least two determinations.

ATP synthase in order to catalyze rapidly and efficiently the process of ATP synthesis in the presence of low concentrations of O_2.

The results presented in Fig. 5 demonstrate that the hypoxia of HA increases the *initial rates* of ATP synthesis regardless of the actual rates of ATP hydrolysis, which strictly obeys Michaelis-Menten kinetics as previously reported (Reynafarje and Pedersen, 1996). In fact, the data show that at ADP concentrations below 30.0 μM, i.e. within the range of the physiological concentration in resting muscle, the *rate ratio between synthesis and hydrolysis of ATP* is significantly higher at HA than at SL. Only at extremely high and nonphysiological concentrations of ADP, the rate-ratio approaches the value of $\sim$1.0 observed under conditions of thermodynamic equilibrium (Boyer, 1979).

The Hypoxic Environment of High Altitude Reduces the Degree of Catalytic Cooperativity During the Process of ATP Synthesis

Although cooperativity was always assumed to be a feature of both ATP synthesis and ATP hydrolysis (Boyer, 1997; Cross *et al.*, 1982; Jeneson *et al.*, 1996; Matsuno-Yagi and Hatefi, 1990; Milgrom and Cross, 1997), it was never before experimentally demonstrated that the thermodynamic correlation between ATP synthesis and ADP concentration has the strict characteristics of a cooperative process. Figure 6 shows that, compatible with the
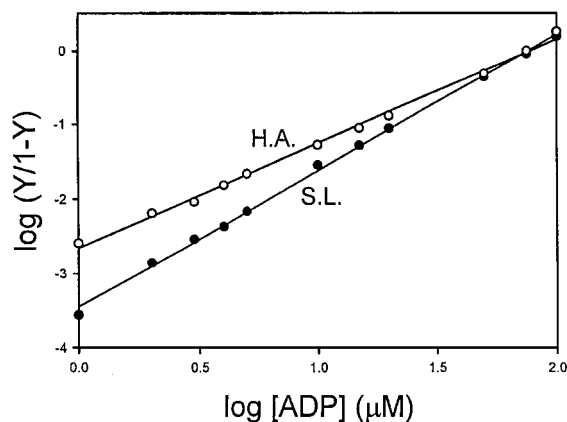
increased levels of both the hypoxia-inducible factor-1 (HIF-1) (Hoppeler and Vogt, 2001) and myoglobin itself (Reynafarje, 1962), the *affinity* of the ATP synthase for ADP is statistically higher at HA than at SL ($p < 0.01$). It is mechanistically significant that although the concentration of ADP at which the catalytic sites are 50% filled is the same in both groups of animals ($P_{50} = 75$ μM), the *fractional occupancy* of these catalytic sites by ADP at concentrations lower than 30.0 μM is much higher at HA than at SL. The extent of *occupancy* of a catalytic site by ADP is related to the degree of cooperativity between catalytic sites, which is significantly lower at HA ($n = 1.36$) than at SL ($n = 1.94$). Thus, Fig. 6 shows that in the presence of only 1.0 μM of ADP, the *fractional occupancy* is three times higher ($Y = 0.06$) at HA than at SL ($Y = 0.02$).

These results indicate that the natural hypoxia of HA evokes crucial transformations in the structure and function of the synthase, increasing the affinity of ADP and decreasing the very high affinity of ATP ($K_d = 10^{-12}$ M) in order to facilitate the synthesis of ATP in the presence of low concentrations of O_2 and ADP (Gnaiger, 2001; Gnaiger *et al.*, 2000).

The Phosphorylation Potential ΔG_P Generated at Low Concentrations of ADP is Higher at High Altitude Than at Sea Level

Data depicted in Fig. 7, constructed with all the data obtained in this study show that in the physiological range
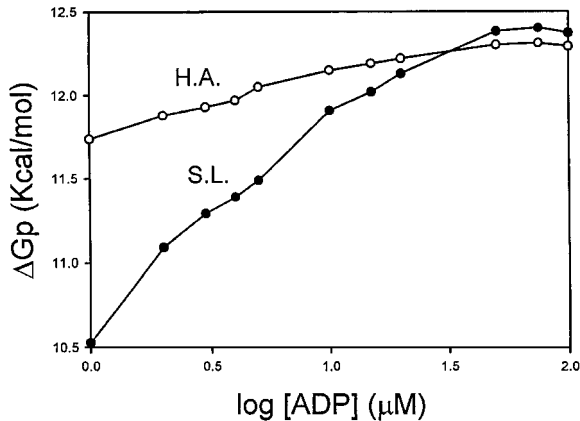
Fig. 7. Effect of the concentrations of O_2 and ADP on the ΔG_P generated by the heart of guinea pigs native to high altitude and sea level. Assay conditions were exactly as described in the legend for Fig. 3. At every ADP concentration below 12.0 μM, the difference in the ΔG_P of these two groups of animals is statistically significant ($p < 0.05$).

of ADP concentrations (from 1 to 30 μM), the ΔG_P is substantially higher at HA than at SL. The maximal values are comparable to those obtained in a reaction catalyzed by both submitochondrial particles and intact mitochondria under conditions of near–thermodynamic equilibrium (Bienfait *et al.*, 1975; Lemasters *et al.*, 1984; Lemasters and Billica, 1981; Slater *et al.*, 1973). It is significant that the ΔG_P generated by the heart of guinea pigs native to HA in the presence of ∼120 μM O_2 is higher than the ΔG_P generated at SL in the presence of twice the concentration of O_2 (∼230 μM). Importantly, Fig. 7 shows that the ΔG_P generated by the heart at HA remains almost unaltered and close to its maximal value (12.3 kcal) when the concentration of ADP increases from 1.0 to 30.0 μM. At SL, on the other hand, the ΔG_P increases 2.0 kcal/mol (from 10.4 to 12.4) when ADP increases in the same range of concentrations. These data show that in the range of physiological concentrations of ADP, the *efficiency* of the heart to utilize the energy of electron transport from succinate to O_2 is 96% of its maximal capacity at HA ($11.8 \times 100/12.3$) and only 84% ($10.4 \times 100/12.4$) at SL.

It is concluded that the hypoxia of HA has fundamentally changed the structure and function of the synthase in order to efficiently and rapidly synthesize ATP in the presence of low concentrations of O_2, saving in this manner a substantial amount of the energy involved in the release of ATP and the binding of ADP (Cross and Duncan, 1996).

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